

2 July 1981

Fighting tsetse flies and people

Opinions differ on the benefits of the transformation of the Punjab in India by the green revolution. Some hold that the transformation of traditional social life has been disastrous. Others, including most of those who live there, hold otherwise. If new strains of wheat have made it possible for India, for the first time in living memory, to be self-sufficient in wheat and thus realistically capable of turning its attention to other than agricultural development, who except those who fear competition in manufactured goods from India can count that a simple tragedy? Is it not, instead, natural to hope that similar transformations will be accomplished in other developing countries? This is the spirit in which, since the early 1970s, the World Bank has been organizing a confederation of donor governments and foundations, known as the Consultative Group for International Agricultural Research, whose frank objective has been to use the model by which the various strains of Mexipak wheat were developed by Dr Norman Borlaug (with imaginative support from the Rockefeller Foundation) to work similar revolutions elsewhere. Inevitably, not all the thirteen research institutes now under the group's wing have been equally successful. The most conspicuous cloud on the horizon is the problem of the International Laboratory for Research on Animal Diseases in Nairobi, now about to appoint its fourth director in seven years.

The Nairobi laboratory (known as ILRAD) has been from the start the odd man out, but for good reason. Its origins lie in the accurate calculation that one of the most serious problems with which African agriculture must contend is that of the tsetse fly. The fact that the trypanosomes for which the tsetse is a vector cause African sleeping sickness among human beings is economically and socially important, but the loss of agricultural productivity because large tracts of Africa cannot be used for growing cattle is a more immediate obstacle to change. So what could be more natural than to set up (in 1974) a research institute for the battle against the tsetse fly? That was the calculation. Broadly speaking, the calculation was correct; a handful of dedicated and able scientists in Nairobi have been able to make substantial progress. There is talk of vaccination (of cattle) and of the development of resistant breeds. The trouble is that the laboratory and its management have been fighting not merely against the tsetse fly but also among themselves.

The most spectacular casualty of this squabbling is Dr A. C. Allison, whose resignation after a dispute with the board of trustees was required just a year ago. Can a laboratory that so lightly dispenses with the services of such a distinguished immunologist have serious work to do? That is what outsiders are bound to think. In practice, the board of trustees has scraped along, appointing an acting director of the laboratory first (for three months from 1 January this year) Dr C. Wells, the new chairman of its trustees, previously the head of the Canadian public veterinary service who is, by common consent, the man most likely to rescue ILRAD, and then Professor R. Zwart from the University of Utrecht, who is also the chairman of the search committee for the new director of the laboratory. Between them, the members of the search committee have recommended to the trustees that the fourth director at ILRAD should be Dr Ross Gray from the University of Edinburgh. At their next meeting towards the end of July, the trustees will be required to decide one way or another.

Trustees of all kinds of bodies are constantly being faced with such dilemmas; which of two proposals to plump for? On this

occasion, the managers of ILRAD should firmly say that they are not ready to make such a simple choice. In due course, to be sure, they may be able to enjoy such a clear-cut luxury. Before that, however, they must understand what has gone wrong at Nairobi. Why have they quarrelled so much among themselves (and why have they nevertheless allowed their numbers to grow)? How does it come about that so much good research has been done at ILRAD while such bitter fights have been carried on? Could it perhaps be that those working at the bench know as clearly as they that Africa will not feed itself until the tsetse fly is beaten? And if so, should they not invest time in putting their structure right? The cause is too good to be neglected.

Loose ends on evolution

Next week the trustees of the British Museum (Natural History) will be meeting and will probably be mulling over, as has been their custom for the past several months, the latest batch of correspondence to appear in *Nature* and elsewhere about the museum of which they are the custodians (and whose name they should change). Some weeks ago (see *Nature* 4 June, p.373), Sir Andrew Huxley was complaining at the centenary celebrations of the "obscurities and irrelevances" of much of this correspondence, but it is improbable that the trustees will feel able to skip that part of their agenda. By way of explanation, Sir Andrew also invited his listeners to reflect that in giving house-room to these supposedly empty but entertaining letters, *Nature* was simply following other commercially-based journals which supply their readers with sensation. (The treatment by *The Times* of London of a recent sensational murder trial was mentioned.) Readers will no doubt decide for themselves whether the charge is justifiable, but that it is based on a scanty knowledge of how the press works is beyond dispute; the most immediate danger for *Nature* is that readers will become so bored that they will take to buying some other journal instead. This is one of the reasons why it has now been decided that the present round of correspondence must cease at the end of this month, whether or not the museum has by then chosen to reply to the issues of substance that have been raised.

Nature's role in this controversy is by no means as malevolent as some at the museum suppose. Like any other journal, *Nature* is to a large extent in the hands of its readers. If people write to say that this or that feature of public policy is mistaken, its first duty is to consider seriously whether what is said deserves a hearing. L. B. Halstead's original complaint that cladism has too great an influence on the museum's exhibition policy (see *Nature* 288, 208; 1980) is interesting and, if true, important. His further claim that cladism is a cloak for Marxism because Marxist-evolutionists tend to be cladists is illogical (and has been disowned) but is nevertheless an interesting observation even if not strong enough to stand on its own; Halstead will no doubt explain what he meant before the month is out. The most remarkable feature of the correspondence that has occupied the past several months is that it has acquired a life of its own. The original issues have been subsumed in others, while questions have arisen about other matters — the physical arrangement of the museum, for example. From time to time, *Nature* has sought to channel the argument, but not with conspicuous success. The relevance of cladistics to evolutionary studies remains unclear; its place in exhibition policy is a puzzle. The issue of punctuated (as distinct



from gradual) evolution has been raised but not resolved (and cannot be as yet). The status of the theory of evolution — metaphysical or otherwise? — is still disputed, but the museum is probably too agnostic on these questions.

The museum in its public role deserves some sympathy. Nobody should underestimate the difficulties of a public institution in mounting a defence against such a variety of charges. The most obvious difficulty is that the scientific members of this important institution differ among themselves both on the weight they give to this or that aspect of evolutionary theory and also in their opinions of the public exhibition policy. There are now suggestions that some of the criticisms that have been raised in correspondence and by *Nature* are concealed attacks on individual members of the museum (and Sir Andrew used the phrase "centering around the scientific thinking of members of the museum's staff"). This is not the case. The most controversial member of the staff, the head of the Public Services Division which is responsible for the exhibition policy, is acknowledged to have a flair for work of this kind, and would presumably plough some other furrow with equal skill if the trustees chose to change course. The trustees' problem is to devise a unified policy in circumstances in which the staff of the museum, distinguished as independent scientists, cannot be expected to toe some well-defined line. The museum should soon pay some attention to the problem (which afflicts all public laboratories) of how to marry the need for some coherent outward face with the inevitable diversity of opinion among its staff. The museum, which courageously has not been afraid to amend the most (and needlessly) tactless part of its newest exhibition, might think of helping other public institutions to a seemingly solution of this increasingly common problem.

Public but going private

Is the British government about to be still more beastly to the nationalized industries it has reluctantly inherited? There are several signs that this may be the case. Last week, the government decreed that the British Gas Corporation (which has for a year been resisting the proposal that it should cease to be a major retailer of domestic gas-burning equipment) should sell its half-share in a small onshore oil field in Dorset, evoking the predictable chorus of anguish from the corporation, which is plainly unsure whether it is a public utility or a kind of conglomerate. A more important development has, however, gone comparatively unnoticed. For now, it seems, the British government is bent on making public agencies in the United Kingdom abandon their long-standing belief that their own definition of their own needs should properly take precedence over the needs of their customers in the marketplace. The document put out by the Department of Industry in mid-June, formally a reply to the perceptive report *Research and Development for Public Purchasing* put out by the Advisory Council for Applied Research and Development more than a year earlier, promises — or threatens — substantial changes in the pattern of British research and development.

The problem is uniquely British. The original report estimated that at least 40 per cent, and perhaps a half, of all applied research and development carried out in the United Kingdom is (or was, in 1975) determined by the needs of British public agencies. The public agencies (government departments, nationalized industries and the research councils) are also eager researchers in their own right; public in-house research and development was running at roughly a third of all British research and development in 1975. A rather greater proportion of defence research and development (some 60 per cent) is put out to private industry on a contract basis than in other fields of the public interest, but this appearance of virtue may be illusory in that the chief beneficiary is the aerospace industry.

The Advisory Council's perceptive complaint, in February 1980, was not that the sums of money involved were too large (which is a different argument) but that government departments

and nationalized industries have wrongly chosen the line of demarcation between themselves and private industry. For, in the most general sense, the objective of most public spending on applied research and development is to define the specification of some product — an aircraft or a repeater in a submarine telephone cable — whose manufacture will afterwards be put out to tender. Manufacturers are always willing enough to put in their bids. Who, after all, wants to turn down business, especially from a customer that can always print the money to pay for what it buys if it cannot otherwise get the funds? The snag, and the chief conclusion of the Advisory Council's report, is that in the cosy relationship that grows up between a paternalistic public sector, willing to shoulder all the cost of research and development itself, and would-be suppliers, scant regard is paid to the long-term interests of the public contractors. Too little of what they make for British government agencies will sell easily elsewhere. Too little of what they spend on research and development goes on the improvement of products that will sell in the wider marketplace.

The Advisory Council's analysis is by no means new. In the 1960s, after a long period of an over-cosy and restrictive relationship with the British Post Office, even the suppliers of telecommunications equipment came to appreciate that the benefits were illusory (yet not a lot has changed since then). The novelty of last year's report was that it advocated a few simple rules of thumb for distributing the balance of research and development between public customers and private contractors. Government and other public agencies have a proper right, even a duty, to carry out research suggesting ways in which the character of their operations may be improved or ways of improving or monitoring the conduct of their existing operations, in matters such as safety. On the other hand, if goods and services are likely primarily to sell to other public customers, the supplier should carry out the necessary research, perhaps with some public help. In intermediate cases, public and private responsibility for research and development should be shared. In general, more publicly supported research and development should be contracted out to private industry.

Rarely can a government's reply to the report of an advisory committee have been as fulsome. With a few justifiable quibbles (where the government is anxious to avoid robbing nationalized industries of their formal autonomy), all the principal recommendations are enthusiastically endorsed. Three government departments (including that for defence, the biggest spender) are said already to have decided to put out more of their research and development. Now the nationalized industries are to be asked to follow suit, but also to find ways in which their own research establishments can sell services overseas. So is a new day dawning for applied research and development in Britain?

The government's enthusiasm is accounted for by its ideology. For months (it seems like years) the government has been talking about "privatization". The Advisory Council's arguments are more objective and thus more persuasive. But everything depends on how effectively the new prescription is carried out. Here doubt persists. The government is probably right not to follow the Advisory Council in the suggestion that there should be an independent board to supervise the fashioning of a new relationship between the public and private sectors in every nationalized industry, but the result will be that final decisions will be made within government departments all too comfortable with past relationships. Conscious as many of the departments will be of the difficulties of managing a substantial shift of resources (for their own laboratories for example) the departments will draw the new lines of demarcation cautiously. This is the respect in which both the original recommendations and the British government's response are inadequate. If the balance of research and development between the public and private sectors is to be shifted substantially, there needs to be some way of anticipating the problems that will arise (such as those afflicting the 75,000 or so people on the government's own research payroll). And there needs also to be, for this purpose and others, some means of letting ministers and taxpayers know what is happening, or about to happen.

White-hot technology lives on in France

New minister plans great leap forward

Paris, June

Part of the mystique of the new Socialist government in France is its almost religious attitude to science, which has startled those on the left who are habitually critical of modern technology. Thus the left-wing daily *Libération* last week described the Minister of State for Science, Jean-Pierre Chevènement, as a man who embraced growth for growth's sake and sought Soviet-style state control of science. There was no room in Chevènement's policies, said *Libération*, for the ecological movement or for the debureaucratization, demilitarization and democratization of science.

This is in part true and part false. Chevènement has lost no time in gathering his powers but he is also calling for greater democracy in science planning going beyond the "democracy of the party faithful". He wants science to be "opened up", but chiefly towards industry. And he is not much interested in the environmentalists. On energy policy he is strongly pro-nuclear. And he will have no control of military science, which absorbs 37 per cent of a total French research and development grant of 50,000 million French francs (£5,000 million).

Chevènement sees science rather in the terms of Mr Harold Wilson's British government of 1964. It is the white heat of the technological revolution in which to forge socialism. Science has an "ethical value", he says, in that "there is no socialism without research". He includes in the term "research" most forms of new knowledge — such as social science, which may see a resurgence.

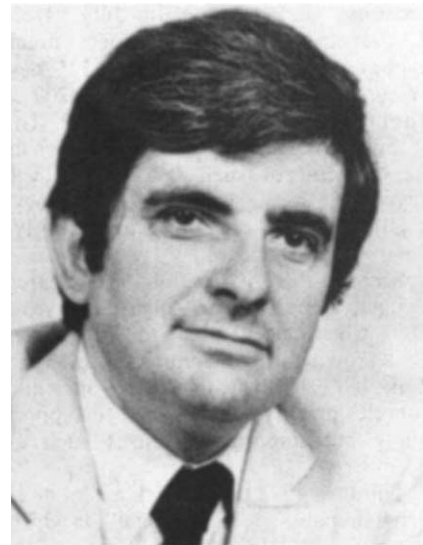
Science in the new government will be expected to help raise the French Gross National Product, to pay for the reconstruction of French society that the Socialist Party has in mind. Some think that an annual growth of 3–4 per cent of the GNP will be needed, compared with 0–1 per cent at present, whence Chevènement's interest in links between science and industry.

During the interregnum, Chevènement battled for weeks with Pierre Joxe, then Minister of Industry, for control of certain key agencies. In the end he appears to have won, although the relevant decree is not yet signed. Joxe has left the government to head the Socialist Party in the new assembly, and the promised decree should give Chevènement the powers he wants.

Specifically, he wants full control of the

Centre National de la Recherche Scientifique (CNRS), the principal body supporting basic science (including social science and humanities) in France; of the Agence Nationale pour la Valorisation de la Recherche (ANVAR), which primes the innovation pump by seeking ideas in universities and government laboratories and supporting their development in industry; of the Mission Interministerielle pour l'Information Scientifique et Technique (MIDIST), an interministerial agency which seeks to improve the national flow of information on science and technology; and of the Délégation Générale à la Recherche Scientifique et Technique (DGRST), which will perform as his administration.

Chevènement (subject to the decree) has also won power over other research agencies which will remain nominally within their present ministries. He will be in control of their budgets, which gives him effective power over their work but not their appointments — a formula he proposed to save ruffled feathers at the ministries he was plundering. These are the Commissariat à l'Energie Atomique (non-military work only), the Centre National d'Etudes Spatiales and the agricultural, medical, ocean science and overseas development research councils. In total,



Chevènement, ready to go . . .

Chevènement will control directly a budget approaching FF 20,000 million.

If the government can hold to its election promises, this budget should rise by some 10 per cent a year until 1985; and by means of low interest loans to industry for research and development projects, the FF 30,000 million industrial sector is planned to rise at the same rate.

For the moment, Chevènement —

Universities jeopardize German budget

A row in West Germany over the funding of new university buildings culminated last week in the rejection of the entire federal budget for 1981. The Bundesrat, the upper chamber of the parliament whose members are the presidents of the eleven Länder, threw out the budget approved on 6 June by the Bundestag, the lower house, on the grounds that it makes insufficient provision for new construction in universities. It was the first time since 1963 that the Bundesrat has overruled the Bundestag-approved budget.

The majority of Länder governments claim that by allocating only DM 680 million for university construction in 1981, compared with the DM 850 million originally planned, the federal government is reneging on its constitutional obligation to meet half of the costs with the Länder. The Bundesrat says that the reduced sum is not enough to pay for projects already under way, let alone for embarking on new ones. Herr Albrecht, president of the Bundesrat, is said to believe that a figure nearer DM 1,300 million is needed if the universities are to expand to meet rising demand. So great is the sense of grievance of the Länder governments that they are claiming that the Bundestag has violated a basic constitutional law — which states that the costs of construction should be shared equally between the Länder and the federal

government — and they are taking their case to the constitutional court.

The matter has also been referred to an arbitration committee which is due to meet next week. An earlier attempt at compromise, which would have guaranteed the Länder reimbursement after 1984 for extra money spent in 1981, failed. But it is now hoped that the Länder will agree to accept reimbursement of DM 120 million in 1982 and the remaining DM 50 million in 1983.

Until this year, cuts in the West German federal budget were practically unheard of. But the worsening economy has forced the government to look for savings. The Länder have used the constitutional obligation on university construction funds to challenge the government's plans for savings and are said to favour cuts in agencies with large bureaucracies such as those dealing with employment and social security.

West Germany has been expanding its universities since the early 1970s and has already spent DM 25,000 million, mainly on upgrading technical colleges to university status. The plan is to invest another DM 20,000 million if the economy can stand it.

The recurrent grant to universities, which is paid by the local Länder governments, is not involved in the dispute.

Judy Redfearn

assuming the decree is forthcoming — has three targets.

First, there is an adjustment to the 1981 national budget, due in July. Here Chevènement does not expect much money, but he has promised 525 new research jobs among his agencies, 200 of them for scientists (the rest for technicians). In the agencies over which he has full control (such as CNRS) he will favour the social sciences, basic biology, medical sciences and (surprisingly) environmental science.

Second, there is the full 1982 research budget, to be fixed in August this year. Here Chevènement plans the full 10 per cent growth, in real terms, and plans to make biotechnology, microelectronics and robotics priority subjects, and to support ailing but important subjects such as chemistry.

Third, in 1982 he will seek a "loi de la programmation de la recherche", in which the National Assembly would vote a firm five-year plan and budget within which his ministry could operate without fear of political setback year to year. Such a "loi programme" has not been tried before outside defence, and it will be a supreme test of Chevènement's undoubted political powers to get such a law approved.

To that end, he is calling a national colloquium of scientists and others interested in science policy for December this year, to thrash out a "new politics" for science and rally support. **Robert Walgate**

US science research

Keyworth reassures

Washington

In his first public appearance since being nominated as President Reagan's Science Advisor and director of the Office of Science and Technology Policy, Dr George A. Keyworth II said last week that the new Administration "views basic research as a vital investment with a good return", that it would seek to increase industry's involvement in research and development and that greater international collaboration was essential in such capital-intensive fields as high-energy physics and space research.

On a less positive note, he warned that the United States could no longer expect to dominate research in all scientific disciplines and should accept that "our country has relinquished its pre-eminence in some scientific fields". He also stressed that support for research should be aimed primarily at those areas considered important for industrial, military or other essential technologies, or where efforts were proving particularly productive, such as computer science and genetic engineering.

Dr Keyworth's remarks were made in a speech to a meeting organized by the American Association for the Advancement of Science (AAAS) to discuss recent trends in the federal research and develop-

ment budget. Many of those present were impressed that although Dr Keyworth, a 41-year-old nuclear physicist who has spent most of his career at the Los Alamos Scientific Laboratory, has virtually no experience of Washington science policy circles, his remarks demonstrated a grasp of many of the issues facing the Administration.

Asked about attempts by the Office of Management and Budget to apply strict cost-accounting procedures to university scientists, for example, Dr Keyworth replied that he himself had never been required to punch a time-clock, but that there were arguments about public accountability that needed to be closely considered. He also expressed concern at the possible damage to international relationships that might follow budget-based decisions to withdraw from projects such as the International Solar Polar Mission, being planned jointly with the European Space Agency.

In his speech, Dr Keyworth emphasized that he saw his role as an "objective adviser" in the White House rather than an inside lobbyist for the engineering and scientific communities. "The Science Advisor in this Administration perhaps more than in any other will be an important member of a *team* of domestic and international advisers", he added.

The content of his speech made it clear that Dr Keyworth is likely to pursue many of the same goals as his predecessor, Dr Frank Press. Referring to the need to reduce the burden of "unnecessary" regulation on the private sector, he said that the Office of Science and Technology Policy would continue to work closely with others in the Administration to strengthen the scientific foundation of federal regulatory decision-making.

However, Dr Keyworth, who has been closely involved in nuclear weapons research at Los Alamos, said he believes that "our country's military might should be second to none", and has also been reported as saying that within the new Administration "science and engineering that support the development of a stronger defence have top priority."

In previous administrations such remarks might have been taken as a warning signal by the biomedical research community. But the commercial potential of genetic engineering, being a rapidly expanding field of high technology, seems to have kept such research in favour. "In general, I think we will see continued strong support for the biological and biomedical sciences", Dr Keyworth said.

Other speakers from the Administration who addressed the AAAS meeting supported the contention that basic science had little to fear from Mr Reagan, and that cuts in the research and development budget were intended to fall primarily in those areas where private industry might shoulder responsibility.

Many of those attending the conference,

however, were not totally convinced. Several questions were raised about the Administration's proposal to eliminate science education programmes at the National Science Foundation (some of which appear to have been salvaged by Congress). Others, such as Dr Ronald W. Lamont-Havers, director of research policy at the Massachusetts General Hospital, expressed concern that even though the Administration might believe in the need to support scientific excellence, budget constraints on research funding were likely to result in high-quality research not receiving adequate federal backing.

David Dickson

EEC university qualifications

Degree of agreement

Brussels

Education ministers from the European Community's ten member states, meeting in Luxembourg in June, considered the tricky problem of improving the mutual recognition of diplomas and periods of study undertaken at higher education institutes.

In theory the principle of the freedom of employment set out in the Treaty of Rome should guarantee that a qualification gained in one member state should open the same economic doors in another state, but this is far from being the case. As one example, a teacher with a degree from the University of Lille who spent her working life in Belgium was not granted her full pension rights because the Belgian state did not recognize her qualification.

No one has been foolhardy enough this time to suggest establishing a Euro-degree on the lines of the much-maligned Euro-loaf or Euro-beer. The low-key approach adopted by the European Commission is to improve the availability of information. EURYDICE, the European information network on education which became operational last year, would link up with national units to provide information in five key areas — entry requirements for first degrees, transfer without loss of acquired rights from a course started in one country to a similar course in another member country, requirements for post-graduate studies or research in another member state, finding work in a member state with qualifications obtained in another, and details of career development.

The Education Council has approved these ideas and the Commission has until March 1982, when the education ministers meet again, to put forward some precise ideas on implementing the scheme.

But this leaves the crucial problem untouched. Should the Community risk an outcry from the academic world by forcing universities to accredit each others' degrees with the same value and worth? Desirable though that would be as a means of increasing the mobility of European

academics, it would ride roughshod over the treasured traditions and autonomy of the institutions.

As a principle or ideal it was welcomed by the ministers and has few opponents in the academic world. The difficulty is that the Education Council realized that the edict cannot be enforced by law. Efforts over the past twenty years to encourage bilateral or multilateral agreements have not kept pace with the growth of higher education and diplomas. The Commission has now been instructed to persevere in its efforts to find ways of encouraging more agreements.

Jasper Becker

High-energy physics

CERN in a hole

The Large Electron Positron ring (LEP), the future project of the European centre for subnuclear research (CERN) near Geneva, has run foul of a small group of environmentalists formed a decade or two ago to protect the Jura Mountains, north of the CERN site, as a national park. Last week the group obtained an injunction at a Lyon tribunal which annuls CERN's licence to bore an exploration gallery for the LEP tunnel into the Jura.

The Lyon decision rests on a technicality. It stipulates that the exploration gallery is not a "temporary construction", as it must be according to the law under which CERN began drilling, but a permanent one. CERN counters that if the tunnel revealed that the construction of LEP would be impossible, then the work would indeed be temporary. However, CERN intends to use the 4 km tunnel as an entrance gallery if LEP goes ahead — so their position was not too strong.

Meanwhile, CERN has not been informed officially of the injunction. Being an international organization, its relations with states — including Switzerland and France — on whose territory it rests, are mediated through the foreign ministers of those states; and there will now be an internal wrangle in France either to get the Lyon decision overruled, or to gain approval for the gallery under another law.

The conservationists, the Association de Protection de la Nature Gessienne (that is, of the Pays de Gex where the gallery was to be drilled), are worried about potential damage to natural springs in the region, and atmospheric pollution from LEP power supplies and cooling systems.

The group timed its injunction well: it coincided with the mid-year CERN council meeting at which national positions on the LEP project were being sounded. In the event, the French delegate at the council, M. Yves Jacques, promised to appeal against the Lyon decision at the highest court — the Conseil d'Etat — and the voting was as previously expected: eight clearly in favour and four undecided. Of

the latter, Denmark expects parliamentary approval soon; after a confusing general election, the Netherlands wishes to delay a decision; Sweden is in similar position after the collapse of the Conservative government; and Norway wishes to wait until after its general election on 14 September.

A committee of the CERN council is to meet in October, and if member states are then ready, a full meeting could be convened to reach the unanimous approval that CERN seeks. (Strictly the eight-to-four majority is sufficient, as LEP will come within the normal CERN budget; but unanimity on such a big commitment — more than SF 600 million (£150 million) for five years or more — is thought politic.) At that final meeting, which could be delayed to December, the central issue is likely to be not whether the states all approve of LEP, but at what budget they want it built — which affects the speed of LEP construction. Argument on the budget was heated in the meeting last week, and no agreement was reached. **Robert Walgate**

Polish economy

Culling ministries

The Polish government's plans for economic recovery will be based on a major restructuring of the country's production ministries, Prime Minister Jaruzelski announced in the Sejm (parliament) recently. During the past few years, there has been a tendency for Polish ministries to proliferate — for example, separate ministries for the metallurgical industry, for the engineering industry, and for heavy and agricultural machinery existed at the same time. Even during the past few weeks, there have been suggestions that some of Poland's most urgent problems, environmental protection and geological surveying among others, could best be tackled by creating new ministries.

The new plan proposes replacing ten existing ministries with four larger ones. These four would cover agriculture, food procurement and production, and forestry; mining of coal and lignite and energy production; heavy industry and machine construction; and chemical processing of timber, natural and artificial fibres, and the ceramics, glass, cellulose and paper industries.

General Jaruzelski went on to remind the Sejm of some of the mistakes of past years, when separating the mining industries from power production, and machine building from foundry production caused serious losses in productivity.

The new proposals have not met with unqualified approval. The Sejm Commission for Forestry and the Timber Industry is unhappy at combining forestry with agriculture, but the corresponding commission dealing with agriculture and the food industry seems fairly happy with the union.

Vera Rich

US standards bureau

Wider horizons

Washington

The US National Bureau of Standards, traditionally a preserve of the physical and engineering sciences, is being asked by a congressional committee to broaden its horizons and take on responsibility for the burgeoning field of biotechnology. The recommendation is one of several being put to the bureau as a way of "enhancing the technological and scientific base of the nation's industrial capability".

There has been little reaction yet from the Reagan Administration to the proposals, which have been drafted by the science, research and technology subcommittee of the House Committee on Science and Technology. Secretary of Commerce Malcolm Baldrige told the committee at a hearing in Washington last week that the whole internal organization of his department, which is responsible for National Bureau of Standards, is under review. He talked of an important role for the bureau — in helping to boost US exports by providing data about foreign product standards but said it was too soon to be specific about changes.

The changes being proposed are in line with the findings of recent studies of the bureau, including reports from the General Accounting Office and the Congressional Research Service. In addition to its traditional responsibilities for weights and measures, several committee members, including chairman Doug Walgren, are convinced that the standards bureau could have a central role in federal efforts to stimulate technological innovation in the private sector.

The bureau's principal responsibility has been to help ensure the compatibility of measurement standards needed by industry, consumers, the scientific community and other organizations. In recent years Congress has given the bureau various additional functions, such as administering the Center for Fire Research and the Center for Consumer Product Technology. But often the bureau has been given a new role without adequate funding with the result that other programmes, have been cut back or eliminated.

Last year both the Senate and the House of Representatives held authorization hearings on the bureau's budget to explore possible remedies. Now the House committee is drafting a bill making the bureau's responsibilities more explicit, but also intended to enlarge its contact with the broader research community.

Many of the committee's suggestions are uncontroversial. There is general agreement that adding the biological to the physical sciences would be appropriate, in particular in the development of standards, testing procedures and measurement instrumentation needed for the advancement of biotechnology and genetic

engineering, although there is concern that the changes should not go too far for fear of conflict with work already being carried out by the National Institutes of Health.

There is widespread support for a proposed amendment that would require the National Bureau of Standards to engage in international cooperation, and that it should maintain measurement research and development as its top priority. The bureau's director, Dr Ernest Ambler, welcomed the committee's recommendation that any new new functions should be provided with adequate funds and manpower.

The committee's suggestion that the bureau be given authority to promote economic growth by "contributing to the development of the basic technologies which underlie product and process development, technological innovation and productivity" raises greater difficulties. Although the bureau does at present support research aimed at the general enhancement of industrial innovation — for example in computers or machine tools — these are undertaken only when they can be shown to be measurement-related. Members of the committee argue, however, that in practice

the bureau already seems to be stretching its definition of measurement in some of the research that it supports. If the organization feels it is limited by the terms of its existing statute, then the solution, they argue, is merely to change the wording of the legislation.

Several witnesses at last week's hearing felt the bureau could have the wider role envisaged in the proposed legislation. It would unduly limit the potential value of the bureau to the nation if its only activities were in the field of metrology.

The committee suggests that the bureau strengthen its links with the academic community by being allowed to make grants and to award fellowships in science and engineering. But Mr William Carey executive officer of the American Association for the Advancement of Science and until recently chairman of the bureau's board of visitors, warned against this on the grounds that "the granting business these days is bogged down with rules and controls for fiscal accountability" and that "adding another granting agency to the confusion would be regrettable".

How far the committee's recommendations go will depend greatly on how far they agree with the Department of Commerce's own views. Mr Baldrige has already eliminated the position of assistant secretary for science and technology, the post previously occupied by Dr Jordan Baruch, a principal author of President Carter's domestic policy review on industrial innovation. Upgrading the National Bureau of Standards would be one way of filling the resulting gap.

David Dickson

Support for Argentinian

Leblon, Brazil

Professor Nicolas G. Bazan, sacked in controversial circumstances as director of the Biochemical Research Institute of the University of the South in Bahia Blanca, Argentina (*Nature* 14 May, p.100), was greeted enthusiastically at a symposium last month organized by the Argentinian Academy of Sciences in the city of Córdoba.

Several of the foreign biologists invited to the symposium, on molecular aspects of the nervous system and memory, carried letters from their professional societies protesting against the dismissal of Bazan and the continued arbitrary attacks by the Argentinian government on scientists. Among them were letters from the American Society of Neurochemistry and from the European Society of Neurochemistry. In spite of the risks of reprisals, the Argentinian academy publicly endorsed these protests from the international scientific community.

Bazan has been invited to visit the United Kingdom to attend the forthcoming biannual meeting of the International Society for Neurochemistry in Nottingham next September, where a special symposium on the human rights issue in Latin America is being planned. It will deal in particular with the situation of the several hundred Argentinian scientists who are reported as "disappeared", "arrested" or "summarily dismissed".

Maurice Bazin

India in space

Another orbit

Lucknow

India's latest venture into space was the launch of the experimental telecommunications satellite, Apple, aboard the third Ariane test flight last month. Apple is unlikely to prove as successful as the Indian Space Research Organisation must have hoped after one of the two solar panels failed to deploy shortly after launch. But there is a chance that such a small satellite, 616 kilogrammes, can operate satisfactorily with one solar panel for long enough for the bulk of the experimental work to be done.

The Apple programme is only one of the fronts on which India is advancing into space. At the end of May, as part of the first developmental flight of its satellite launch vehicle SLV-3, India placed a 38-kilogramme satellite into a low Earth orbit. That was the second satellite to be put into orbit using the Indian rocket, the first having been launched last July (*Nature* 286, 324; 1980).

The latest Indian-launched satellite, designated RS-DI, carried a solid-state

sensor capable of identifying salient features on the Earth and transmitting data back to the ground stations.

The main objective of the Apple satellite launched on Ariane but built at the Space Application Centre in Ahmedabad, is to gain experience in the operation and management of communications satellites. Yet another satellite, Bhaskara II, is due for launch at the end of the year and is for the development of remote sensing technology.

Experiments planned for Apple include logging the movements of railway wagons and of railway passenger reservations in large cities, the setting up of an inter-library information system and the development of a "national classroom" for postgraduate engineering students at the five Indian Institutes of Technology. Under the last programme, students at the five centres will be given televised lectures and be able to question the lecturers directly.

Zaka Imam

Occupational health

US industry to pay

Washington

In a decision that represents a victory for the US labour movement and a major blow to the Reagan Administration's efforts to reduce the burden of health and safety regulations on private industry, the US Supreme Court has ruled that the Department of Labor's Occupational Safety and Health Administration (OSHA) is not required to carry out rigorous cost-benefit analyses of new regulations covering exposure to toxic substances before the rules are put into effect.

The drive to put occupational safety and environmental regulations on a strictly cost-benefit footing has been central to the new Administration's "regulatory reform" strategy. In line with this, the head of OSHA, Mr Thorne Auchter, asked the Supreme Court in March to delay a decision on whether regulations introduced by the Carter Administration in 1978 to reduce workers' exposure to cotton dust were invalid since they had not been accompanied by a thorough analysis of the relative costs and benefits.

However, the court has turned a deaf ear to the agency's request. In a move that will now require both OSHA and the White House to ask for new legislation from Congress if it wants to pursue the strategy, the court ruled by five votes to three, with one abstention, that there is nothing in the law to require such analysis. Rather it has accepted the interpretation of OSHA officials under the previous administration, that the mandate requires the agency to do all it can to reduce health hazards to workers, short of bankrupting the industry concerned.

The new cotton dust regulations, which are already being implemented by most large textile manufacturers, are aimed at

lowering the incidence of byssinosis (brown lung disease) among the nation's 800,000 textile workers. In 1978 OSHA issued regulations requiring the permissible level of cotton dust to which workers could be exposed to be reduced from 1,000 to 200 microgrammes per cubic metre of air.

Even at the time the ruling was highly controversial. Whereas OSHA officials estimated that the cost of compliance to industry would be about \$650 million, industry itself put the figure at \$2,700 million.

The Supreme Court's decision is the final step in a legal challenge launched by the American Textile Manufacturers Association, asking that the rules be disregarded because the agency had not demonstrated that the expected benefits outweighed the likely costs of implementation. If the appeal had been sustained, it would have allowed the Administration to proceed relatively unimpeded with its plan to apply strict cost-benefit analysis to various regulations already in effect — for example those covering exposure to arsenic and lead — with a view to reducing their severity.

This strategy will have to be reassessed, now that the Supreme Court, in a ruling by Justice William J. Brennan, has concluded that OSHA is not required to carry out such an analysis, but merely to determine that its regulations are technically and economically feasible.

Although professing to welcome the justices' verdict, since it formally supports the government's position, OSHA officials also acknowledge that it is a set-back to their attempts to chart a new course for the agency that would be more congenial to private industry. Mr Auchter has already made clear his strong disagreement with the philosophy and policies of his predecessor, Dr Eula Bingham.

Predictably the court's verdict has been warmly welcomed by labour unions representing textile workers, but their jubilation may be premature. Already OSHA officials have revised their approach to new regulations in the light of last July's ruling by the Supreme Court, in rejecting proposed new standards for exposure to benzene (*Nature* 286, 97; 1980). And following the cotton dust decision, a spokesman for the textile manufacturers said that the companies "continue to believe, as does the Administration, that cost-benefit analysis is an important element in achieving rational and cost-effective rule-making".

But much of the fire has disappeared from the textile industry's complaints. Many companies acknowledge that their calculations of likely costs were based on the conversion of old equipment and that new regulations have led them to buy new machines that have already produced significant gains in profitability. It is a silver lining that few would have expected when the regulations were hailed as a large black cloud over the industry

David Dickson

Dietary vitamin C Bigger daily dose?

Britain's answer to Linus Pauling now argues that the government should raise the recommended daily amount of vitamin C from 30 mg to 100 mg — enough to saturate body tissues with the vitamin, but nowhere near the "megadose" levels Pauling advocates.

The new British "Mr Vitamin C" is a mild-mannered Welshman, Dr R. Elwyn Hughes of the University of Wales Institute of Science and Technology. His book on the subject has just been launched by the British Nutrition Foundation, which though supported entirely by the food industry is at pains to provide "balanced" information on nutritional matters. The foundation supports Dr Hughes's recommendation that the government should reassess the criteria by which recommended daily amounts are arrived at, but makes no commitment to the actual level of vitamin C we need. On this Dr Hughes — officially — stands alone.

We owe the British recommended amounts to a group of conscientious objectors who in 1940 were subjected to zero vitamin C diets. They soon showed the symptoms of scurvy, and their intake of vitamin C was raised slowly until the scurvy disappeared. It disappeared at 10 mg a day; so the government tripled this amount to allow a margin of error.



Britain's Pauling?

However, the past decade has seen the discovery of useful functions for vitamin C other than just preventing scurvy, says Dr Hughes, such as involvement in lipid metabolism and atherogenesis; brain metabolism; carnitine metabolism (which relates to muscle fatigue and lack of stamina); resistance to infection; and the detoxification of potentially harmful drugs, including certain tranquillizers. Although such evidence is incomplete it is sufficient to commend tissue saturation levels of the vitamin, he concludes.

According to British dietitians, the average daily intake of vitamin C in the United Kingdom is 60 mg, equivalent to the

content of two fresh oranges. But many people take much less. In a survey of old people's homes in South Wales, Dr Hughes discovered average intakes of less than 20 mg, and in some cases less than 10 mg.

Dr Hughes does not recommend megadosage, however. Its benefits are unproved, he says. Half of any excess over saturation is swept out in the faeces, and the rest rapidly in the urine; and moreover there is evidence of disadvantage, in particular that toxic metals can be forced across the placental membrane by high vitamin C fluxes.

Robert Walgate

Soviet mineral supplies Digging deeper

Talk in the West of a "resource war" between the Soviet Union and the United States seems to have prompted a reaction from the Soviet Minister of Geology, Evgenii Kozlovskii. Talking on Radio Moscow, he said that speculation that the Soviet Union would soon become a net importer of mineral ores was mere Western propaganda.

The minister's statements may have been a response to the recent meeting of geologists and Soviet watchers at the Johns Hopkins University (see *Nature* 11 June, p.444), which concluded that while there was little evidence to support the view of some of President Reagan's more "hawkish" advisers that the Soviet Union was deliberately waging a resource war, there did appear to be production difficulties in the Soviet Union leading to a cutback in mineral exports and the stepping up of imports.

According to Kozlovskii, the Soviet Union can not only meet its own mineral requirements, but also has surpluses for export. Although his assertion that the Soviet Union has the world's largest known deposits of coal, gas, iron, manganese and apatite is probably true, it does not follow that sufficient supplies are available to Soviet industry.

At present, apparently, Soviet geologists are discovering new mineral deposits faster than the mining engineers can extract them. But there is still great emphasis on new methods of surveying for minerals, including satellite surveys and the drilling of deep and "super-deep" boreholes.

Deep boreholes, in fact, are to play a big part in Soviet geophysical research in the 1980s. The first such hole, on the Kola peninsula, reached a depth of 10,800 metres and a new hole to be drilled in Western Siberia is thought to be of particular significance because of hopes of finding oil deposits in Pre-Jurassic strata at depths of between 5,000 and 7,000 metres. Even deeper drillings are being planned, to depths of greater than 13,000 metres, where high ambient temperatures (up to 350°C) and high pressures will require new drilling techniques and monitoring instruments.

Vera Rich

CORRESPONDENCE

Defence for Chelsea

SIR — Your correspondent, in summarizing the recommendations of the Committee on Academic Organisation of London University, has failed to comment on an alarming feature of the discussion document. The committee state that their judgements on academic standards in institutions are based on "general reputation" and their own personal opinions. It is on this basis that it is implied that the majority of departments at Chelsea College are not of the standard of the university at all. How else can those who survive the proposed "peer review" be accommodated on a "single site", given the present size of the college?

The University of London is a great, but complex, institution. It faces brutal cuts in funding over an alarmingly short period of time and it was thus wise to have set up the Committee on Academic Organisation under Sir Peter Swinnerton-Dyer to assess the implications of the expected cuts and advise on their implementation with minimal damage to academic standards. But the committee has yielded to the temptation of extrapolating objective financial analysis into arbitrary recommendations based on subjective and highly damaging prejudgements. Instead of setting the scene for cooperative rationalization and contraction, the document has generated anger and dissension.

The committee had already clearly established that for years the sub-allocation of the UGC block grant to London by the Court of the University has favoured certain colleges at the expense of others. Inevitably, the high unit-cost colleges, with their favourable facilities and staff ratios, have tended to attract more research grant monies than have the low unit-cost colleges. Nevertheless, and in spite of the conditions under which they have been forced to operate, the under-provided schools have not only become better integrated and more innovative than the giants, but contain many departments of true distinction.

Yet the committee's conclusion is that it is "unthinkable" that the necessary savings should be made where the high costs have been identified. Instead, the worst of the burden is to fall on colleges that, having been starved from the centre, are now judged, *a priori*, not to be academically excellent — as if, in any case, excellence can ever be ascribed to a college rather than to individual departments.

Chelsea College has survived a succession of externally-engineered crises, and in the face of them has grown from strength to strength. Operating at exceptionally low unit-costs we still have built up departments that are among the biggest and best of their kind in the university, and some of which have a major international reputation. Why then have we been singled out for gratuitous attack? Is it simply and cynically that we are a sitting target, fully-stretched financially and spatially, and big enough if destroyed to spare others their share of the agony?

A simple way to destroy a college is to malign its reputation, so that potential students and benefactors and the various grant-giving bodies shy away. If this is not what the committee consciously set out to do, then the publication of the discussion document represents a sad error of judgment.

The attack on academic standards at Chelsea College not only offends us, it insults

boards of studies of the university and its external examiners, jointly charged with maintaining standards in their subjects. They are, by implication, accused of incompetence.

The committee should now retract their scenarios and their gratuitous insults to Chelsea and to the other colleges judged to be "weak", and leave the schools themselves voluntarily to suggest rationalization within objectively defined guidelines. Otherwise, the university should forthrightly reject as incompetent the latter part of the report.

H. BAUM

Department of Biochemistry,
Chelsea College, London SW3, UK

Postdocs are OK

SIR — I object strongly to your comments regarding the future for postdoctoral fellows in the United States (*Nature* 11 June, p.441). The 50 per cent increase in postdoctoral grants since 1972 represents an expansion of the equality of opportunity for recent graduates, and the federal policy-makers responsible ought to be applauded, rather than attacked with words like "demeaning employment", "exploited", and "scandal". You confuse an equality of *opportunity* with an equality of *outcome*. Sadly, this is a common confusion that muddies the minds of the liberal thinkers of both our peoples, and breeds frustration amongst the unfortunate victims that subscribe to their thought.

The value of postdoctoral study and its "prize" of an academic post remain intact ("88 per cent of the most recently appointed assistant professors in chemistry had done a postdoctoral stint" etc., p.443). For many, this study is preceded by an overkill of classroom exercises and examinations, and followed by endless faculty committee meetings and governmental paperwork, leaving only those precious postdoctoral years where one can devote full time to research, and *attempt* to achieve the scholastic maturity necessary for undertaking truly independent investigations. (Incidentally, independence is the mark of a "professional", not the social status, health insurance, or salary implied by your comments and the juvenile anecdotes of the disappointed. Anything less denotes hired help, regardless of training, qualification, or special ability.) The statistics of the Grodzins Committee fail to take into account those students of highest ability and fortune who achieve this maturity while earning their degree (all are supposed to) and therefore do not need a postdoctoral period of additional opportunity. For the remaining majority, it stands to reason that an increase in the numbers electing to enter this race for a fixed number of prizes will be followed by an increase in the numbers who do not win. Is that so surprising as to deserve front page coverage? Is it morally offensive? Is it indicative of someone in authority having failed in their responsibility, as you state? I think not. You propose that those who do not win "should be paid a bounty, a kind of retrospective recompense for deprivations". Now *that* I do find morally offensive, lying somewhere between race-fixing and squandering public money on a losing proposition, or to be blunt, on losers.

DEXTER B. NORTHROP

University of Wisconsin-Madison, USA

Cox sure?

SIR — Barry Cox's comments on the British Museum (Natural History) exhibit on Origin of Species (*Nature* 4 June, p.373) contain an incredible statement which must not pass without challenge. Otherwise, the creationists' claim that evolutionary science is really dogma will have received the *imprimatur* of your journal. The statement is:

"We [biologists] don't even think that it [the evidence] could support a dramatically different *scientific* (*sic*) theory, in the way that earlier observations of the heavens were transformed from being compatible with an Earth-centred Universe to demonstrating a Sun-centred Solar System."

As a practising biologist, I wish to register my dissent. Surely, Dr Cox got carried away. Does he understand the implications of novel findings in genetics so completely that he can make such a statement with serenity? Is it certain that new data on genome organization and variation will not lead to fundamentally new ideas about the mechanisms of speciation? Have we fully assimilated the lessons of overlapping and interrupted coding sequences, mobile genetic elements, and somatic differentiation by chromosome rearrangements? With all due respect to Dr Cox and the many scientists who believe that the problem of evolution is solved "in principle", let me state my conviction that there is a great deal of aptness in the analogy between Ptolemaic astronomy and our current understanding of evolution.

JAMES A. SHAPIRO

Department of Microbiology,
University of Chicago, Illinois, USA

Fit for what?

SIR — In his article (*Nature* 4 June, p.373) on the new Origin of Species exhibition in the British Museum (Natural History), Dr Cox quotes the sound track of a film loop "The Survival of the Fittest is an empty phrase, it is a play on words". This appears to refer to the widespread belief that "survival of the fittest" is a tautology, because our only measure of the fitness of an organism is its ability to survive. This is not so; in the long run survival is a problem for palaeontology, because fossils are our only evidence of what has failed to survive. And in palaeontology, there is a technique which can be used to estimate fitness (in the ordinary dictionary sense). This uses the "paradigm" method developed by Rudwick¹ for testing functions inferred from structures in fossils. The structure concerned is compared with a paradigm which is the ideal for the performance of that function (due allowance being made for the nature of animal materials). Paul² has taken this farther, and compared with the appropriate paradigms a series of structures which perform the same functions in different primitive echinoderms. He concluded that, for the functions of protection, feeding and respiration, the echinoderms of the Cambrian and Ordovician were less efficient than their successors. Moreover, this was compatible with the elimination of the unfit by competition (which is "survival of the fittest" in reverse).

Continued on p. 95

NEWS AND VIEWS

Modern light microscopy

from Michael Spencer

NEW techniques in electron microscopy have over the years been matched by innovations in light microscopy, which retains the unique advantage of being applicable to living cells. Two recent papers illustrate this well, and also emphasize that in several respects the conventional formula for the resolving power of a light microscope may be said to underestimate the potential of the instrument.

The first, by Daniel Axelrod (*J. Cell Biol.* **89**; 141, 1981), is about a new application to microscopy of total internal reflection fluorescence (TIRF), a branch of spectroscopy dating from the sixties. It complements another existing technique (also introduced about twenty years ago) called interference reflection microscopy, which can show up cell-substrate contacts as darker regions against a background of light reflected from other areas of cell surface. In the case of TIRF, however, the opposite situation applies: only the contact areas give rise to light collected by the objective.

Light from a laser (Fig. 1) enters a glass or fused quartz cube at such an angle that it undergoes total internal reflection at the interface between a glass coverslip and a film of medium containing cultured cells. The cell membranes have previously been 'doped' with a fluorescent label. Although geometrical optics predicts no transmission of light across the interface, electro-

magnetic theory shows that a disturbance which Axelrod calls an evanescent wave (a term taken from diffraction theory) will penetrate into the liquid medium. This wave propagates parallel to the surface with an intensity that decays exponentially with perpendicular distance from the interface; although in the ideal theoretical case no energy crosses the boundary, many observers (from Newton onwards) have shown that this is not true in practice. Within a thin layer adjacent to the interface, fluorescent molecules can be excited by the evanescent wave.

The depth d at which the wave's intensity falls to $1/e$ of the incident value depends on the angle of incidence; the larger this angle, the smaller the value of d . It is possible to arrange that most of the fluorescence is confined to a layer only about 100 nm thick, which is considerably less than the wavelength of the light; even smaller values would be feasible with the use of high-refractive index components. The technique allows membrane-bound receptors, or elements of cytoskeletal structure, to be selectively labelled and shown up without interference from fluorescence arising elsewhere in the specimen. Furthermore, by varying the angle of incidence (and hence the depth of excitation) one can map the topography of the membrane facing the substrate. Figure 1(c) illustrates the kind of picture that is obtained.

The second paper concerns the application of polarizing microscopy to biological systems. Shinya Inoué is the doyen of workers in this field, and has for many years been engaged in pushing the technique to the limits set by current technology. Visitors to the inner sanctum where he conducted some of his experi-

ments were required to exchange their shoes for carpet slippers, lest particles of New England dust should contaminate the optical surfaces and give rise to unwanted scattering. His latest report (*J. Cell Biol.* **89**; 346, 1981) concerns the improvements now possible with the use of video image processing.

The most serious problem in observing weakly birefringent objects in a polarizing microscope is that the method is a dark-field one; very little light is collected, and even if the dark-adapted observer sees something clearly, it can require a long photographic exposure to record the image. Furthermore, the background level is raised (and contrast reduced) by polarization effects arising at lens surfaces. Inoué tackled this problem more than twenty years ago and showed that it could be ameliorated by the use of 'rectified optics' incorporating additional elements. However, this still does not enable one to use the highest-quality objectives that are essential for achieving maximum resolution, because these incorporate fluorite lenses that contain crystalline inhomogeneities whose effects cannot be compensated for.

With video recording and reproduction, however, it is simple to subtract an unwanted background level electronically, and thus enhance the contrast. With a highly sensitive video camera one can also

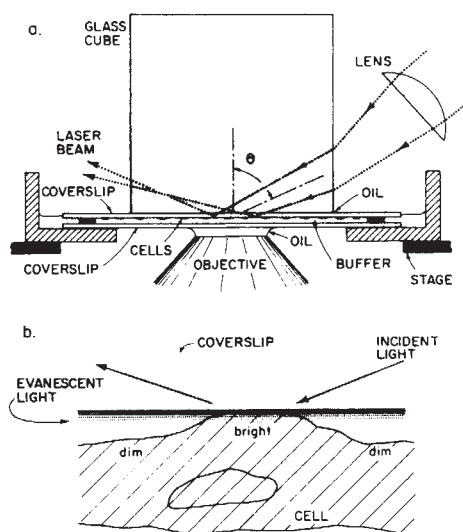


Fig.1 TIRF microscope apparatus for viewing cells in culture. (a) The optical system on the microscope stage; (b) magnified diagram of the evanescent wave at the coverslip/solution interface, exciting fluorescence of those portions of a cell in close contact with the coverslip. (c) Human skin fibroblasts labelled with a fluorescent marker and illuminated by TIRF. Angle of incidence 74.3° , $d = 105$ nm. Bar = 30 nm. From Axelrod, D. J. *Cell Biol.* **89**, 141 (1981).



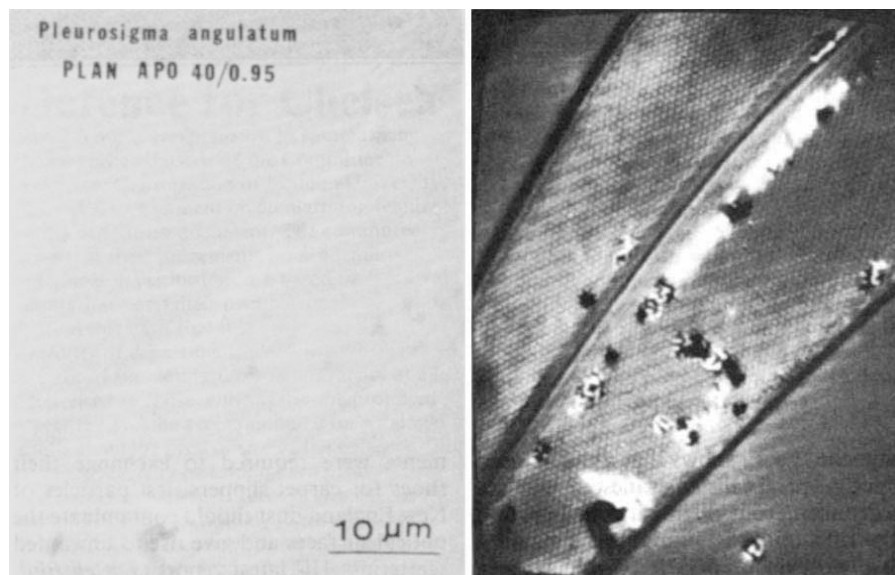


Fig.2 Appearance of a diatom in video-polarized light microscopy, using a plan apochromatic objective. The left scene was adjusted to match that seen by eye through the ocular, while the right scene was electronically adjusted for optimum contrast. The diatom was embedded in a medium of nearly matching refractive index. From Inoué, S. *J. Cell Biol.* **89**, 346 (1981).

reduce the exposure time for photography, so that rapidly moving objects can be followed. Just how striking the improvement can be is illustrated by Fig.2, which compares a diatom as seen by eye through

the microscope with the image photographed from a video system adjusted for optimum contrast. Comparable improvements are also possible in the field of interference contrast microscopy.

Inoué's paper includes pictures, taken by the new technique, of beating cilia, acrosomal processes in the act of extending and rotating bacterial flagella. All of these have diameters much smaller than a wavelength of light. Dark-field photography of sub-microscopic objects is not, of course, new, but the use of polarized light adds new information about the orientation of macromolecules within the structures; in particular, birefringent structures show up without interference from the non-birefringent background. Inoué illustrates this point with a high-resolution picture of the ridges on the surface of an epithelial cell.

He adds a caveat that to get the best results one must take account of the non-ideal performance of both optical and video components, and exercise great care in optimizing the illumination. The light levels are so low that by maladjustment one can easily lose the image in a background of instrumental noise. Such problems, and the rival merits of different commercial systems, are the subject of hot debate among the cognoscenti; there is no doubt, however, that future advances in video technology will give further boosts to the older branches of microscopy. □

Michael Spencer is a Research Fellow in the Department of Biophysics at King's College, London.

Seabirds and oil: the worst winter

from Chris Mead and Stephen Baillie

LAST winter more oiled seabirds bearing rings were reported to the British ringing office than ever before: a total of 103 birds of ten species from December to March. Many were found in areas where exceptional numbers of dead and dying oiled birds had been counted on beaches (see the figure). A single incident in the Skagerrak accounted for 22 ringed birds and resulted in a total of 30,000 oiled seabirds being found on beaches. Other areas where recoveries of ringed birds were concentrated suffered oil pollution from a variety of sources rather than the acute pollution from a single source experienced on the Skagerrak. The aggregate of beach counts of oiled birds in Western Europe last winter probably approached 60,000 (ref. 1).

The lack of comparative figures from earlier years for counts of beached birds over all the affected areas precludes the use of such counts for annual comparisons. However, details of ringing recoveries for many years are available and allow the scale of the oiling mortality of British populations to be assessed. The worst affected species, the two large auks guillemot (*Uria aalge*) and razorbill (*Alca*

torda), spend a large part of their time swimming and are therefore likely to encounter floating oil^{2,3}. These two species accounted for 84 per cent of the oiled ringed birds. Information on the numbers of ringed birds at risk and numbers recovered from last winter and 1967–68 to 1978–79 is given in the table. This shows that guillemots experienced four times as much oiling as usual and razorbills about three times as much. For guillemot, but not for razorbill, more birds were also 'found dead' than usual. These figures conceal some age-related differences between the two species. For guillemot the youngest birds were worst affected (4.5-fold mortality increase) with immatures suffering a 2.3-fold increase but adults only 1.4 times normal mortality. For razorbills only adults were badly affected (2.5-fold increase in mortality) with younger birds surviving as well as or better than normal. These results reflect the species' different migration patterns^{4–6}: most sub-adult razorbills winter well south of the areas of oiling and adult guillemots mostly winter close to the breeding areas which were unaffected by oil.

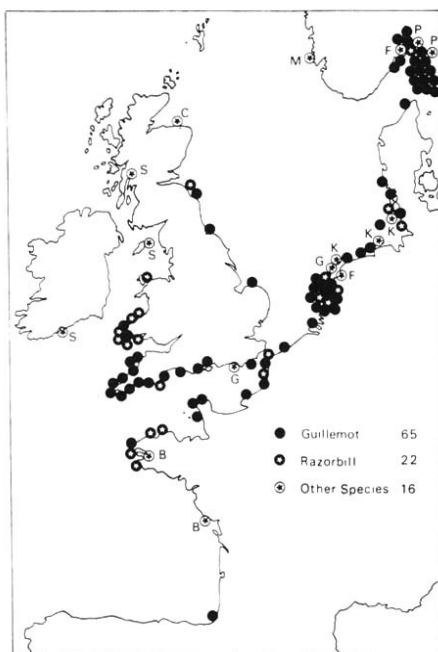
Although several thousands of each species are marked each year, the ringing effort is not uniformly distributed throughout the birds' breeding range in

Britain and Ireland. There are four areas where it is possible to speculate about the effect of the 1980–81 mortality of guillemots: Orkney and Shetland, Grampian region, north-west Scotland and Saltee, Co. Wexford. If there is no compensatory increase in the survival of the remaining birds and the age of first breeding does not change, any excess mortality during a single winter might affect breeding numbers in three ways: immediately, by the loss of adults from the breeding population; annually for four years, by the loss of immature birds in second to fifth winters; and after four years by the loss of first year birds now entering the breeding population for the first time.

When some allowance has been made for the variation in ringing effort, it seems likely that the guillemots from eastern areas have suffered worst. The Orkney and

1. Mead, C. *OTO News* **112**, 1 (1981).
2. Andrews, J.H. & Standring, K.T. *Marine Oil Pollution and Birds* (RSPB, 1979).
3. Mead, C.J. & O'Connor, R.J. *Seabird Ringing Recoveries and Oil Pollution*. (Evidence to Royal Commission on Environmental Pollution — BTO, 1980).
4. Mead, C.J. *Bird Study* **21**, 45 (1974).
5. Lloyd, C.S. *Bird Study* **21**, 102 (1974).
6. Birkhead, T.R. *Bird Study* **21**, 241 (1974).
7. Birkhead, T.R. & Hudson, P.J. *Ornis Scand.* **8**, 145 (1977).
8. Seabird Group *Seabird Counting Manual: Auks* (1980).
9. Stowe, T.J. *Ibis* (in the press).

Chris Mead and Stephen Baillie are research workers at the British Trust for Ornithology, Tring.



Finding localities of oiled seabirds ringed in Britain and Ireland and reported from December 1980 to March 1981. Black circle — guillemot (*Uria aalge*: 65 records). White star — razorbill (*Alca torda*: 22 records). Black star — other species: 16 records keyed as follows: (F) Fulmar (*Fulmarus glacialis*: 2); (G) Gannet (*Sula bassana*: 2); (C) Cormorant (*Phalacrocorax carbo*: 1); (S) Shag (*Phalacrocorax aristotelis*: 3); (M) Red-breasted merganser (*Mergus serrator*: 1); (B) Great skua (*Stercorarius skua*: 2); (K) Kittiwake (*Rissa tridactyla*: 3); (P) Puffin (*Fratercula arctica*: 2).

Winter mortality, December to February, for 1980–81 compared with 1967–79 for British and Irish ringed guillemots and razorbills

	Guillemot (<i>Uria aalge</i>)		Razorbill (<i>Alca torda</i>)	
	1980–81	1967–79	1980–81	1967–79
Ringed birds at risk†	18,000	83,000	15,500	122,500
Recoveries reported:				
Oiled	61	68	20	55
'Found dead'††	20	56	4	84
Total	81	124	24	139
Recoveries/1,000 at risk:				
Oiled	3.4 *	0.8	1.3 *	0.4
'Found dead'	1.1	0.7	0.3	0.7
Total	4.5 *	1.5	1.5	1.1

* indicates significantly higher rate than for the other period with probability < 0.001

† The figure for birds at risk has been calculated from the ringing totals reported over the last 25 years (1980 figures provisional) with allowance made for first year mortality (50 per cent), annual immature mortality of 16 per cent and adult mortalities of 6 per cent for guillemot and 9 per cent for razorbill^{4–7}.

†† 'Found dead' recoveries include only beached birds not reported as oiled. Birds known to have been shot, snared or caught during fishing operations have been excluded throughout.

Shetland guillemot populations will be reduced by 2 per cent immediately, followed by 1 per cent per annum for three more years and a further 4–5 per cent in 1985 when the 1980 cohort should start to breed. It is possible that the guillemots from Grampian colonies may have suffered as badly. However, the adults and immatures from north-west Scottish colonies seem to have survived better but the increased mortality of first year birds will affect breeding numbers in four years. In this area 5 per cent of the breeding razorbills may have been lost. Saltee results indicate that neither species suffered particularly high mortality last winter.

Even the largest predicted annual

decreases in populations (about 5 per cent) are probably too small to be detected by current census techniques⁸. Recent breeding population studies indicate that population changes on this scale (or slightly greater) have affected many auk colonies in Britain and Ireland over the last decade^{7,9}. However, this oiling mortality, if repeated over several winters, would result in a steady decline of guillemot and razorbill populations over much of northern Britain. Together with the threat of inshore oil exploitation, all those responsible for the conservation of seabirds, particularly in Scotland, have a very real cause for concern. □

Moonquake meanings

from Peter J. Smith

As the Earth has earthquakes, so the Moon has moonquakes; and insofar as events of both types have definite foci, generate seismic waves and radiate energy, they are clearly recognizable as belonging to the same class of phenomena. Thereafter the resemblance weakens, for the distributions of lunar and terrestrial shocks in both space and time are quite different. Whereas most earthquake epicentres lie along narrow bands coincident with major crustal features, moonquake epicentres exhibit no such correlation. Whereas earthquake foci lie at depths of 0–700 km, their counterparts in the Moon mostly occur in the depth range 700–1,100 km (deep moonquakes), although there are also a few at less well determined depths of rather less than 200 km (shallow moonquakes). And whereas earthquakes taken together demonstrate no clear temporal patterns,

deep moonquakes have a strong periodicity of 27 days. Lunar quakes also differ from the terrestrial variety in that they occur repeatedly at a limited number of foci.

The reason for all these differences is evidently the influence of different tectonic styles, the importance of which is re-emphasized by new calculations of lunar seismic energy and moonquake source parameters carried out by Goins *et al.* (*J. geophys. Res.* **86**: 378, 1981). This is not the first time that an attempt has been made to determine the seismic energy released by moonquakes, although Goins and his colleagues claim, certainly correctly, that previous estimates were unsatisfactory in that they failed to take sufficient account of such factors as instrument frequency response and the effects of intense scattering near the lunar surface. On the other hand, it is not entirely clear that many of the conclusions drawn by Goins *et al.* could not equally have been drawn from the earlier calculations, notwithstanding that they apparently underestimated

seismic energy by a factor of about 100 and moonquake magnitudes by a factor of about 2. But that is no argument against having the correct, or at least more 'realistic', figures at last; and in any case Goins and his co-workers have also made the first determinations of additional source parameters such as seismic moment and stress drop.

According to the new figures, then, the 500 or so deep moonquakes with body wave magnitudes (m_b) greater than 1.6 (maximum $m_b = 3.0$) recorded annually by the Apollo Passive Seismic Net release energy of 8×10^{13} ergs a year. So where does this energy come from? Or to put it another way, what is the basic mechanism for the generation of deep moonquakes? Comparison with the Earth is no help here, for deep moonquakes have no terrestrial equivalents. What is relevant, however, is the fact that the marked periodicity of 27 days in the event occurrence frequency at each deep moonquake focus exactly matches the dominant 27-day period of the

tide induced in the Moon by the Earth. Moreover, the number and size of deep moonquakes in any given month also correlates with the 206-day variation in the lunar tide resulting from the perturbation of the Moon's orbit by the Sun. The occurrence times of deep moonquakes are thus evidently controlled by tidal forces; and since the tidal energy dissipated in the Moon is 10^{21} – 10^{22} ergs a year, this source is obviously more than sufficient.

But the tidal energy dissipated in the Earth is, at 10^{27} ergs a year, even greater than that dissipated in the Moon. Why, then, are there apparently no tidally induced earthquakes? For this interesting conundrum Goins and his colleagues offer three explanations, not necessarily mutually exclusive. One is that terrestrial lithospheric stresses due to lunar forces are about an order of magnitude smaller than the lunar stresses due to Earth forces at moonquake depths. The second is that there may indeed be some tidal earthquakes but that most, if not all, of them are masked by tectonic earthquakes and microseismic noise. And third, the 'dynamic character of the Earth's interior' generally precludes the development of long-term strain buildup at terrestrial depths of greater than about 700 km.

The latter two explanations thus bring us directly to tectonic style, which seems to be much more important than the relative magnitudes of possible driving forces when it comes to a planet's seismic characteristics. For example, the thermal energy available in the Moon (2×10^{26} ergs a year) is not much less than that in the Earth (9×10^{27} ergs a year), but the different sizes of the two bodies lead to different distributions of temperature with depth and hence to different tectonic regimes. The result for the Earth is a thin lithosphere, lithospheric breakup, plate motion and a dominant near-surface seismic distribution generated tectonically. On the Moon, by contrast, the lithosphere is much too thick to fracture; and the temperature–depth distribution, and hence the shear modulus–depth variation, is such as to concentrate the tidal stress into a particular depth range. Thus whereas heat is the chief fundamental influence on terrestrial seismicity, the corresponding lunar influence is tidal.

Not that things can be quite as simple as that. Although tidal stresses vary smoothly over the lunar surface, moonquake epicentres do not. Most of them lie on the Moon's nearside, although that could merely reflect the difficulty of detecting farside events with nearside seismometers. But of greater significance, perhaps, is the highly uneven distribution of epicentres on the nearside, and in particular, a marked disinclination of epicentres to lie in the south-east quadrant of the face. This could be simply a matter of chance; but it might also suggest that, notwithstanding the dominance of tidal forces, the precise locations of foci within the 700–1,100 km

layer are governed by pre-existing inhomogeneities or zones of weakness.

Then there are those shallow moonquakes, hitherto mentioned but otherwise ignored. Although the passive seismic net has only been recording about five of these a year with m_b greater than 2.2, their maximum magnitude (4.8) is much greater than that of deep moonquakes (3.0) and the total seismic energy released by them annually is 2,500 times greater (2×10^{17} ergs

a year). Yet they demonstrate no obvious periodicities in their times of occurrence, are not related to any obvious surface features, and do not occur repeatedly at the same foci. Moreover, although they release far less energy than even intraplate earthquakes (about 3 per cent of terrestrial seismic activity), their stress drops are comparable with, and sometimes even higher than, those of most terrestrial events. [

Europe regains the lead in atomic physics?

from M.R.C. McDowell

THE First European Conference* on Atomic Physics (including Molecular Physics) shattered the belief that the United States necessarily leads the world in any branch of physics. This resurgence in Europe is not confined to a few countries — important new advances were reported from Poland and Spain, for example.

The charge exchange reaction ($X^{q+} + e + Y^{p+} \rightarrow X^{q+1} + (Y^{p+} + e)$ in which an electron is transferred from one ion (or atom) to another, is attracting much theoretical and experimental interest. One reason is that it is now clear that charge exchange-assisted recombination can move the ionization equilibrium in thermonuclear plasmas several stages of ionization away from the 'coronal equilibrium' values. At impact velocities V greater than the orbital velocity of the captured electron, energy and momentum are transferred with the electron, and the corresponding momentum transfer factors in the expression for the cross-section can greatly reduce the rate of the process. The process leads to highly specific final states.

A. Macias and A. Riera (Universidad Autonoma de Madrid) have clarified this momentum transfer problem by showing that the translation factors introduce a new class of coupling which may be more important than dynamical coupling in determining the final state. Further advances in our understanding of charge exchange came from J. H. McGuire (Hans-Meitner Institute, Berlin) who, with his student P. R. Simony (Kansas State University), has shown that the ν^{-11} dependence of the proton–hydrogen charge exchange cross-section is manifested in a secondary maximum in the differential cross-section, and this was confirmed in a different theoretical

approach by A. Salin (University of Bordeaux), who finds an interference dip in this secondary peak. However, H. Knudsen (Aarhus, Denmark) claimed that her group had not seen this interference experimentally. L. Dubé and J. S. Briggs (Freiburg, Germany) have shown that in capture from one excited state to another, the second Born contribution is dominant over the first Born at high impact velocities, and capture occurs into final states with maximal overlap with the initial state. It became clear in discussion that no one knows the exact velocity dependence, at high velocity, of any charge exchange process.

Theoretical advances also dominated the discussion on the behaviour of atoms in very intense electromagnetic fields, the latter being of great interest in the astrophysics of White Dwarfs and Neutron Stars, as well as in the ablation of inertial fusion targets. Fields of interest range up to 10^{13} G, although currently available laboratory fields are restricted to about 4×10^5 G. P. Cavaliere, C. Leone and R. Zangara (University of Palermo, Italy) have shown that the normal double-loop pattern observed in electron impact triple differential ionization measurements splits into a quadrupole pattern when a laser beam, at right angles to the electron beam, also interacts with the target. In the field-free case the transition is $s \rightarrow p$, but with the field on, it may be $s \rightarrow s$ or $s \rightarrow d$, the electron emitting or absorbing one photon. The quadrupole pattern appears to be associated with absorptions [$s \rightarrow (p+1)$]. They also show that the process of laser-assisted electron impact ionization of atoms can lead to appreciable laser gain. F.

M.R.C. McDowell is Professor of Applied Mathematics at Royal Holloway College, University of London, and was assisted by both D.N. Stacey, Clarendon Laboratory, Oxford and G.V. Marr, University of Reading in preparing this report.

*The Conference, sponsored by the Atomic Physics Division of the European Physical Society, was held at the University of Heidelberg (April 6–10 1981), and was organized by Professor G. zu Putlitz. The two-volume Book of Abstracts (eds J. Kowalski, G. zu Putlitz, H. G. Weber) is published in *Europhysics Conference Abstracts*.

M. Faisal (University of Bielefeld, Germany) with A. Larni and N. K. Rahman (University of Pisa) have demonstrated theoretically that laser gain should also be exhibited in laser-assisted recombination. Detailed calculations for recombination to He (5^1P) suggest that high gain can be obtained at low laser intensity if the electrons are highly monochromatic. Photoionization of atomic hydrogen in intense magnetic fields has been calculated by S. M. Kara (British Nuclear Fuels), who finds intense resonances at the Coulomb-modified Landau levels. An exact scaling theorem for the isoelectronic sequence has subsequently been given by G. Wunner, H. Ruder, W. Schmitt, H. Herold (University of Erlangen, Germany) and M. McDowell (Royal Holloway College, London University), which reduces to a simple scaling in the dipole approximation. This should be experimentally testable, using a synchrotron as a light source.

For field-free photoionization, U. Heinzmann (University of Munster, Germany) followed up his earlier work on production of spin-polarized photoelectrons by reporting absolute measurements of the eigen-quantum defects in Ar, Xe and Kr from threshold to 40 eV, which join on smoothly through threshold with the bound-bound transition values of n^3 times the oscillator strengths $f_{nn'}$ from level n to level n' . This is very direct experimental evidence for the validity of Seaton's multichannel quantum defect theory, which is of considerable astrophysical importance. (For Xe, see U. Heinzmann *Appl. Opt.* **19**; 4087, 1980.)

Dramatic developments were reported in the study of nuclei by optical spectroscopy. Two experimental advances, the tuneable laser and on-line mass separation, have allowed the charge radii, spins and moments of long sequences of radioactive nuclei to be measured. In particular, the variations in charge radius (giving rise to so-called 'isotope shifts' in atomic spectral lines) have given a great deal of nuclear structure information over the years, but until quite recently very little work had been done on light elements or unstable nuclei. Suddenly, vast quantities of data are appearing, largely due to the efforts of two groups (based at Mainz and Orsay) working at the ISOLDE mass separator at CERN. Rubidium, for example, has two naturally occurring isotopes; results for over 20 isotopes are now available, including several isomers. They show, remarkably, that the nuclear size actually decreases as the neutron number rises from 40 to the shell closure at 50; there is then a sharp increase, much more rapid than is predicted by the usual nuclear models.

Other elements show equally interesting features, the most striking so far being the shape isomerism found in neutron-deficient mercury isotopes.

The results are a valuable testing ground for nuclear theory. The variations in

nuclear size show broad overall features which should lead to a better insight into the systematics of nuclear behaviour, while the finer details, for example isomer shifts, depend sensitively on the structure of the individual nuclei concerned.

The work of V. S. Letokhov (Academy of Sciences, Moscow) on the resonance interaction of laser radiation with single atoms now allows the simultaneous availability of high spectral resolution with the ultimate in sensitivity and detection selectivity in that he claims to be able to detect and do physics on one atom in 10^{12} . High Rydberg atomic states have now been demonstrated to be very attractive for high-resolution spectroscopy and metrological work (H. Walther, Munich). Exciting a single Rydberg state by laser opens the possibility of higher-resolution measurements of the fundamental constants. Beam foil spectroscopy continues to be used and recently has allowed the

observation of radiative transitions between bound states of the negative ion of lithium (I. Martinson, Lund).

The study of molecular systems is entering a new and exciting phase. New avenues of experimental study are being opened up by the multichannel quantum defect theory (M. J. Seaton, London; Ch. Jungen, Orsay), typified by work reported by S. Krümmacher (Freiburg) using the Orsay synchrotron light source to study the inner shell photoionization of CO. It is important to note that Heinzmann's direct verification of this theory allows absolute calibration. High-resolution spectroscopy of Cl_2 reported by J. Le Calvé (Saclay), threshold electron spectroscopy (M. Kurepa, Belgrade) and resonance fluorescence (T. Möller, Hamburg) on the same molecule show that there is much which can be obtained by application of both new and traditional techniques to the analysis of individual molecular systems. □

A gradient of membrane protein in the retina

from Colin J. Barnstable

THE MECHANISM by which cells sense and signal their position within an organ or organism remains one of the least understood problems in biology. Many experimental systems such as chick limb development, hydra regeneration and insect cuticle formation have indicated that gradients of positional information exist and can be sensed and acted upon by the relevant cells. Both the nature of these gradients and the sensing mechanisms are unknown. In the nervous system, the position of an individual neurone has important consequences for the nature of its synaptic input and the target it innervates. For example, motor neurones in the developing spinal cord migrate to particular positions and the resulting clusters of cells can be shown to be committed to innervate a particular muscle¹. Innervation is only achieved after the growing axons reach this correct target some distance away. In this system the mechanism that specifies which motor neurones will innervate which muscles, and the mechanisms by which the growing axon reaches its target, remain unknown.

This general problem of neural specificity has also been intensively studied in the visual system and particularly in the topographical projection of retinal ganglion cell axons to the optic tectum. One current debate centres around the mechanism by which this ordered

Colin J. Barnstable is Instructor in Neurobiology, Harvard Medical School.

projection is formed. In some fishes it may be the result of a maintained topography in the optic nerve². In the adult cat³, on the other hand, the ganglion cell axons are mixed up in the optic nerve and sort out at their targets. This implies that some form of 'chemoaffinity' between axon and target cell exists. Models for this have varied from a strict 'lock-and-key' relationship between each ganglion cell axon and its tectal target neurone to a quantitative gradient or gradients of interaction molecules arranged according to particular retinal axes⁴.

These interaction molecules have usually been studied in terms of selective cell adhesion using methods developed for studying the aggregation and sorting of a variety of embryonic tissues (see ref. 5 for a review). In the retino-ectal system it has been shown that in general cells from a given portion of retina preferentially adhere to cells from the tectal area that includes their normal synaptic target. From studies such as these it has been concluded that there are several interacting gradients that can be used to specify cell position and subsequent interaction. None of these studies, however, has successfully identified the molecular nature of these gradients.

A recent paper by Trisler, Schneider and Nirenberg⁶ has demonstrated that a gradient of concentration of a particular molecule does exist across the avian retina. To demonstrate this Trisler *et al.* looked

for, and found, a monoclonal antibody that bound more strongly to dorsal retina than ventral retina. When 14-day chick embryo retinas were cut into eight segments along a dorsal-ventral axis and assayed for antibody binding, the most dorsal segment bound 35-fold more antibody than the most ventral. Presumably the extremes of the gradient show a much greater difference. The antigen detected by the monoclonal antibody has been named TOP, after 'toponymic' which the authors define as 'a marker of position'. It appears to be a cell surface protein since it can be assayed on living cells, is heat labile and is trypsin sensitive. Immunocytochemical localization of antigen in 14-day chick embryo retina showed that it was present in all layers and, therefore, possibly in all cell types. The gradient is thought to represent differences in the amount of antigen per cell rather than regional differences in cell or membrane concentration since mechanically dissociated cells gave different intensities of immunofluorescence according to the part of the retina chosen. Essentially all the features of the gradient were found at every age tested from 4-day old embryo to adult.

Whatever the mechanism for generating the gradient, simple 'organizing' or 'controlling' areas outside the retina seem not to be involved. The evidence for this comes from an unusual embryo found by Trisler *et al.* that had a third eye in the middle of the forehead facing in a dorsoanterior direction. Even though the eye was in an abnormal orientation, the gradient of TOP antigen showed a normal orientation with respect to its own dorsal-ventral axis defined by the choroid fissure.

The importance of this paper for biology in general is that it has demonstrated a molecular gradient in a particular organ. Its importance in aiding our understanding of neural specificity will depend upon whether the cells within the retina or in the optic tectum use the information contained in the gradient for their physiological interactions. □

1. Landmesser, L. *J. Physiol., Lond.* **284**, 391-414 (1978).
2. Scholes, J.H. *Nature* **278**, 620-624 (1979).
3. Horton, J.C., Greenwood, M.M. & Hubel, D.H. *Nature* **282**, 720-722 (1979).
4. Sperry, R.W. *Proc. natn. Acad. Sci. U.S.A.* **50**, 703 (1963).
5. Moscona, A.A. in *Neural Recognition* (ed. Barondes, J.S.) 205 (Plenum, New York, 1976).
6. Trisler, G.D., Schneider, M.D. & Nirenberg, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2145 (1981).

Galactic nuclei

from R.A.E. Fosbury

SINCE the upsurge of interest in Seyfert galaxies and the discovery of quasars in the early 1960s, galactic nuclei have been prime targets for the application of new observational techniques. The more active galactic nuclei have not disappointed observers as their high luminosity extends over the whole electromagnetic spectrum. The current status of research on galactic nuclei, both active and 'normal', was examined by the participants at a recent conference* at the Royal Greenwich Observatory.

In the spiral Seyfert galaxies, the nucleus has a profound influence over the physical conditions in a region of typical diameter one kiloparsec (3×10^{21} cm). The optical observers call this the narrow line region (NLR) since it is the source of the strong forbidden emission lines with widths of order 300 km s^{-1} characteristic of this class of object. The region is also now recognised to be a source of radio radiation with a relatively steep spectrum. Andrew Wilson (University of Maryland) reviewed new radio observations, primarily from the Very Large Array (VLA) in New Mexico, which showed the Seyferts to be intermediate between normal and radio

galaxies. Indeed, some show a double or triple structure on a scale of a few arc seconds, reminiscent of a classical double radio source but prevented from bursting out of the immediate nuclear environment by a spiral of dense interstellar medium. Wilson noted an interesting lack of correlation between radio and optical axes in the Seyferts which may suggest that the nucleus is not influenced very much by what the rest of the galaxy is doing. Spencer, Boller, Steward and Lonsdale (Jodrell Bank) complemented the VLA work by showing similarly high-resolution radio maps made at lower frequencies with the new multi-telescope radio-linked interferometer which spans a significant fraction of the western side of Britain. Because of the steep spectra, this instrument is particularly suited to studies of the nuclear surroundings. The small angular size subtended by the nuclei makes it difficult to reconstruct the geometric and kinematic state of the various components. It has long been realised that the emission line profiles are a rich source of such information, although difficult to interpret. Mark Whittle (Institute of Astronomy, Cambridge) reported an extensive study of the profiles originating in the NLR and at last offered some hope of being able to distinguish

between inflowing and outflowing material from careful comparisons of the asymmetries seen in lines from different ionization states. Although it is generally agreed that most of the emission line radiation emitted by active nuclei is the result of photoionization processes, there is still considerable uncertainty whether the origin of the ionizing continuum is non-thermal with a power-law spectrum or a combination of this with thermal radiation. Shock processes have been advocated for some nuclei which do not show a strong continuum. Even for these, Daniel Péquignot (University of Meudon) illustrated the versatility of photoionization models to explain some of the low-ionization spectra. His models produce the strong auroral line with regions of high density and reproduce the optical spectra quite satisfactorily using a two-component ionizing spectrum; the approach was, however, running into some difficulties with the UV emission line observations.

Bernard Pagel (Royal Observatory, Herstmonceux) considered the elemental abundances of both normal and active nuclei. With the caveat that the abundance determination methods used for nuclei are somewhat unreliable, it is perhaps surprising that activity seems to have little effect on chemical composition. In fact, all the observed galactic nuclei seem to have very similar abundances and, in particular, there is no evidence for secondary processing in nuclear HII regions as the nitrogen to oxygen ratio is consistent with that of oxygen to hydrogen. How normal are 'normal' galactic nuclei anyway? Robert Sanders (University of Groningen) sketched a picture of recurrent activity in which gas clouds within the inner few hundred parsecs lost a small fraction of their mass to a black hole on each collision and produced one event of about 10^{56} ergs every 10^7 years. He supported the thesis with reference to our own Galaxy by pointing out the expanding ring seen in molecular emission lines which should oscillate with about that period. New X-ray observations of our own nucleus were presented by Brin Cooke (University of Leicester). The orbiting Einstein Observatory has shown a number of hard-spectrum point sources together with a diffuse component extended along the galactic plane. One of the (non-variable) point sources is coincident with the radio source Sagittarius A west and he pointed out that the radio/X-ray flux ratio is about the same as that in the radio galaxy Centaurus A. Willem Wamsteker (VILSPA) described some optical observations of the nucleus of the nearby spiral galaxy M83 which showed non-circular motions very similar to those seen in our Galaxy.

The second half of the conference collected together important observations

R.A.E. Fosbury is at the Royal Greenwich Observatory, Herstmonceux Castle, UK.

*The 25th Herstmonceux Conference was held at the Royal Greenwich Observatory in April, 1981.

of the very innermost parts (< 1 parsec) of active nuclei, known generally as the broad-line region (BLR). In a theoretical preview, Andy Fabian (Institute of Astronomy, Cambridge) set the size scales of the various components and suggested that the presence of an X-ray emission line due to iron may depend on the (variable) continuum luminosity of the nucleus through the influence of the ionization parameter. This may explain why the Ariel V satellite had seen the line in NGC 5548 while Einstein had not.

Eugen Preuss (University of Bonn) showed the sophisticated level attained by radio VLBI techniques. In particular, the use of large telescopes as interferometer elements, for example, by Effelsberg and Westerbork, had resulted in sensitivity levels of just a few milli-Janskys ($10^{-29} \text{ W m}^{-2} \text{ Hz}^{-1}$) at a frequency of 5 GHz. A recent observation had detected the nucleus of the nearby and well known Seyfert NGC 4151 on a scale of 20 milliarc-seconds but with a flux of only 25 mJy at 5 GHz.

Suzi Collin-Souffrin (University of Meudon) and Hagai Netzer (University of Tel Aviv) discussed the emission line spectrum from the BLR. Collin-Souffrin stressed the importance of an exact treatment of radiative transfer and pointed out that the broad-line clouds look very different when viewed towards or away from the ionizing source. Netzer encouraged the observers to produce more good measurements of the strong emission line fluxes in Seyfert galaxies, particularly in the ultraviolet.

Jaqueline Bergeron (University of Paris) devoted attention to those Seyfert galaxies which show the permitted spectrum of singly ionized iron. She demonstrated that the resonance line emission in the ultraviolet was not well correlated with the optical subordinate lines. The UV lines were always present while it is well known that the optical lines are absent in many objects, particularly those with strong radio emission.

Toon Snijders (University College, London) reported the very extensive UV observations of line and continuum variability of NGC 4151 since February 1978. The most rapid continuum variations yet found show a doubling time as short as six days. The emission lines are also seen to vary and lag behind the continuum; the CIV emission broadens when the nucleus is stronger. In addition, the absorption lines are found to vary. Very similar results were reported by Jean Clavel (University of Meudon) for another Seyfert galaxy — NGC 4593. In some cases theorists will soon be driven to make time-dependent photoionization models for the BLR.

Michael Penston (Royal Observatory, Herstmonceux) described the class of absorption line thought to arise in material very close to the nucleus of some active galaxies. The best known examples are Markarian 231 and NGC 4151, though they

are seen also in about 10 per cent of quasars. The lines are broad and always blueshifted with respect to the narrow emission lines. The galaxies with absorption show a range of polarization properties but tend to be more highly polarized than other active nuclei except for the 'violently variable' cases.

The short wavelength end of the spectrum has great advantages for the study of active nuclei and Richard Mushotzky (Goddard Space Flight Center) left the audience in no doubt that post-Einstein X-ray astronomy had much to say on the subject. The use of the X-ray object as a 'white'-light source gives information

about the size of the broad-line clouds, their covering factor, element abundances and the overall shape and temperature structure of the region. X-ray variability is seen in many objects, the most extreme example being NGC 6814 where the time scale may be as short as 100 seconds. It is notable that short-timescale variability is confined to the lower-luminosity objects. Going to even higher energies, Tony Dean (University of Southampton) reported Balloon γ -ray observations of NGC 4151 and MCG 8-11-11. The Seyfert spectra appear to turn over near 3 MeV which is different from the behaviour of the cosmic diffuse background. □

Tyrosine phosphorylation and oncogenes

from Peter Newmark

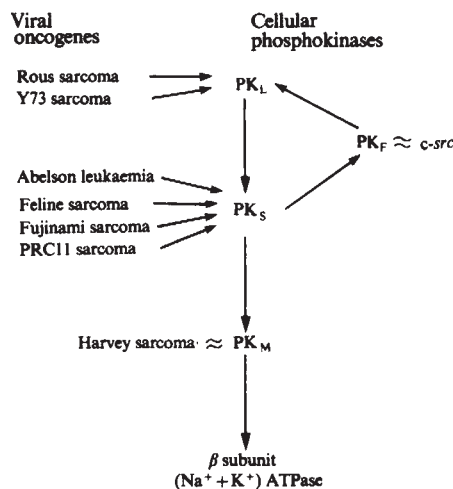
PROVOCATIVELY placed on the front cover of the abstract book from this year's RNA Tumor Virus meeting at Cold Spring Harbor* is the scheme of a biochemical pathway (shown in modified form below) that was, without doubt, the major talking point of the meeting. For, if the pathway is substantially correct it brings a whole new perspective to the actions of RNA tumour viruses and, conceivably, to how the products of their transforming genes convert a normal cell into a tumour cell. So remarkable would this be that the general reaction of those at the meeting was to breathe in sharply and start counting to ten, the number of months it could take for the work to be substantiated.

The basis of the work has been the discovery by M. Spector, working with Professor E. Racker, at Cornell University, of a cellular cascade of protein kinases. Activation of the cascade by phosphorylation of a tyrosine residue of any of its members results in phosphorylation and inactivation of plasma membrane ($\text{Na}^+ + \text{K}^+$)ATPase — which is how the story began. Last year, Spector *et al.* published two preliminary papers. The first (*J. biol. Chem.* **255**; 5504, 1980) reported that the efficiency of Na^+ pumping by the ($\text{Na}^+ + \text{K}^+$)ATPase of Ehrlich ascites tumour cells was much lower than normal; the second (*J. biol. Chem.* **255**; 8370) claimed, on the basis of some remarkable reconstitution experiments, that the inefficiency was due to phosphorylation of a tyrosine residue of the β subunit of the enzyme by a newly discovered protein kinase, now known as PK_M .

More recently (*J. biol. Chem.* **256**; 4219, 1981) the same group reported that PK_M is

the substrate for another new protein kinase, PK_S , which, in turn, is phosphorylated by yet another new kinase, PK_L . Each of these kinases had been purified to homogeneity and they had been shown to be structurally dissimilar by immunological criteria and by peptide mapping. Speaking at the meeting, Spector (Cornell University) added a fourth new kinase, PK_F , to the cascade and produced evidence that each kinase was only active in the cascade when phosphorylated on tyrosine (and not when phosphorylated on serine or on both serine and tyrosine).

Of more relevance to the topic of the meeting, Spector described how members of the cascade are themselves the substrates for phosphorylation, on tyrosine, by the products of the transforming genes of



Arrows indicate tyrosine phosphorylating activity. The product of the Harvey sarcoma viral oncogene is closely related to the regulatory subunit of PK_M ; c-src, the cellular homologue of the Rous sarcoma viral oncogene, is closely related to PK_F .

*RNA Tumor Viruses 20–24 May 1981.

RNA tumour viruses. It is at this point that the whole scheme touches ground with the well established fact that the products of the transforming genes of several RNA tumour viruses can phosphorylate tyrosine groups. The substrates for this phosphorylative activity have so far been confined to a variety of cellular proteins which, apart from vinculin (B.M. Sefton *et al. Cell* 24; 165, 1981), are identified only by molecular weight. Many of the transforming gene products are themselves phosphorylated on a carboxy-terminal tyrosine which is adjacent to a glutamic acid (H. Opperman, University of California, San Francisco; J.C. Neil, University of Southern California).

Spector also reported that the product of the transforming gene of Harvey murine sarcoma virus is immunologically related to the regulatory subunit of PK_M and that the product of the cellular gene (*c-src*), from which the transforming gene (*v-src*) of Rous sarcoma virus is thought to have been derived, is very similar to PK_F both immunologically and by the criterion of peptide mapping. Finally, Spector noted that he had observed some, but not necessarily all, of the differences between the phosphorylation of proteins in normal and transformed cells predicted by his scheme.

The cellular homologues of the oncogenes of RNA tumour viruses provided another focus of the meeting. Recently published evidence (W.S. Hayward *et al. Nature* 290; 475, 1981) shows that avian leukosis virus, which lacks a transforming gene, can induce lymphomas in chickens by increasing the transcription of *c-myc*, the cellular homologue of the transforming gene of the avian acute leukaemia virus, MC29. H.-J. Kung (Michigan State University) reported that reticuloendotheliosis virus, although genetically unrelated to avian leukosis virus, also becomes integrated next to *c-myc* in the bursal lymphomas that it causes in chickens.

The question of the mechanisms by which the transcription of *c-myc* is increased in these lymphomas has recently been considered in these columns (J. Wyke *Nature* 290; 629, 1981) and was much discussed at the meeting. The simplest proposal is that the virus inserts one of its own promoters (contained within the LTR — the 'long terminal repeat' — sequence located at each end of the integrated viral genome), correctly oriented and positioned to act as a promoter for the transcription of *c-myc*. There is good evidence for this mechanism in many lymphomas (Hayward *et al. op. cit.*).

But there are exceptions. Some of these must be the result of the viral activation of *c-myc* by less straightforward and still mysterious mechanisms since they follow the insertion of an avian leukosis virus either in the wrong orientation or the

wrong position (3' instead of 5') to act as a simple promoter of transcription (G. Payne, University of California, San Francisco). Analogous evidence comes from the demonstration that it is sufficient to link the LTR sequence of the Moloney murine sarcoma virus to the 3' end of either the oncogene of the virus or its cellular homologue for either of these hybrids to be able to transform NIH 3T3 cells *in vitro* (T.G. Wood, NIH). The latter observation very strongly supports the notion that cellular oncogenes live up to their presumptive name.

Although nobody reported an attempt to detect increased transcription of human cellular oncogenes (the latest of which is reported on p.31 of this issue of *Nature*) in human tumour cells, A. Eva (NCI) presented the results of a search for transcripts related to MC29, simian sarcoma virus, Abelson murine leukaemia virus and BALB-murine sarcoma virus of human tumour cell lines. Such transcripts were often present, sometimes several of them, and in considerable amounts. On the other hand, there was considerable variation between different cell lines of the same tumour type; furthermore, normal human fibroblasts also had low levels of some of the same or similar transcripts as the tumour cells. It is therefore still too early to know what, if any, bearing these transcripts have on the transformed phenotype of human tumour cell lines, let alone tumours themselves.

Cellular oncogenes are becoming ten a penny (or, better, a dime a dozen in view of their predominant discoverers) and several new ones were reported. The most interesting were the two cellular oncogenes in the rat, described by D.R. Lowy (NCI), which are related to the viral oncogenes of the Harvey murine sarcoma virus. One of them had no intervening sequences and a restriction map identical with that of the viral gene. The other had intervening sequences — as do nearly all other cellular oncogenes (for example, D. Shalloway *et al. Cell* 24; 531, 1981; and R. Dalla Favera, this issue of *Nature*, p.31). If either was ligated by its 5' end to a LTR of the Harvey murine sarcoma virus, the resulting hybrid DNA could transform 3T3 cells. The cells transformed by the unsplit gene synthesized a protein that was indistinguishable from the transforming protein of the virus; the cells with the split gene synthesized a protein that closely resembled a protein of the same size found in small quantities in normal cells.

Two other aspects of the meeting warrant a brief mention. One was the production of a complete sequence for the Moloney murine leukaemia virus (T.M. Shinnick, Scripps Clinic) and two slightly different, complete sequences for the Moloney murine sarcoma virus (C. van Bevern, Salk Institute and E.P. Reddy, NCI). With these sequences it was possible to see exactly which parts of its genome the leukaemia virus had to sacrifice in order (as

one supposes) to accommodate the cellular oncogene and so become the sarcoma virus. Also available, hot out of Harvard and discussed in part by D. Schwartz, was the complete sequence of Rous sarcoma virus. A striking feature of both the Rous and the Moloney murine sarcoma viruses was that their *pol* and *env* genes overlapped in different frames. Also striking was the fact that the *gag* and *pol* genes of Rous sarcoma virus were in different frames, implying that splicing must occur to generate the messenger for the *gag-pol* protein.

The other focus was the development of cloning vectors from tumour viruses. In theory, at least, such vectors will be highly efficient and will stably integrate a single copy of the gene they are carrying into the host genome. Spleen necrosis virus (K. Shimotohno, University of Wisconsin) and Harvey murine sarcoma virus (E.M. Scolnick, NCI) have already been used as vectors for herpes virus thymidine kinase gene. And as Scolnick pointed out, it is also possible to utilize the hybrid virus to study, more closely, the function of the viral oncogene, using thymidine kinase as the selectable marker. Who knows, that may be a fruitful way to get a new foothold on the Spector cascade. □

100 years ago

A Private visit was paid on Saturday last to the Channel tunnel experimental works by Sir E. Watkin, M.P. (chairman of the South-Eastern Railway Company), and a large party of scientific and other gentlemen. Very satisfactory progress was found to have been made at Abbot's Cliff since the last visit. The heading has now been advanced a total length of nearly half a mile. The tunnel is kept well free of water, and a good average rate of work is maintained. The work at the new shaft at Shakespeare's Cliff promises to be even more satisfactory. A very superior boring machine is used, and a more powerful engine is being fitted up to drive it.

The first part of a fourth edition of Griffith and Henfrey's useful Micrographic Dictionary has appeared. It is expected that the issue will be completed in twenty-one of these monthly parts, which will include important additions representing recent scientific progress. This work is known to aim especially at helping the microscopic observer to discover what any object is which may be presented to him, and by the aid of the Bibliography to refer to more extensive treatises for further details. A system is also adopted by which one is guided to a general knowledge of particular departments of science. There is an introduction on the use of the microscope. Dr. Griffith is assisted in the editing by the Rev. Mr. Berkeley and Prof. Rupert Jones.

From *Nature* 24, 7 July, 225, 1881.

ARTICLES

Lucifer dyes—highly fluorescent dyes for biological tracing

Walter W. Stewart

National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205, USA

Lucifer dyes are intensely fluorescent 4-aminonaphthalimides which are readily visible in living cells at concentrations and levels of illumination at which they are nontoxic. Because of their low molecular weight they frequently pass from one cell to another; this widespread phenomenon, termed dye-coupling, is thought to reveal functional relationships between cells. Lucifer dyes can also be used for ultrastructural tracing by comparison of electron micrographs with light micrographs of the same thin section. In addition, they show promise for backfilling neurones through cut nerves, for visualizing the results of retrograde axonal transport and for the covalent labelling of macromolecules.

SINCE their introduction in 1978 Lucifer yellow CH and Lucifer yellow VS have been used with considerable success as intracellular markers in a wide variety of biological systems. Both dyes contain the same sulphonated 4-aminonaphthalimide moiety, but they differ in the substituent on the imide nitrogen (Fig. 1). Their syntheses, to be reported elsewhere, are based on the commercial wool dye brilliant sulphoflavine (CI 56205). The dyes have similar spectral properties: absorption maxima at 280 nm and 430 nm, corrected emission maxima near 540 nm, and quantum yields of about 0.25. The high quantum yields make possible the detection of the dyes at low concentration, and the wide separation between the absorption and emission maxima facilitates excitation at one wavelength and observation of fluorescence at another.

The dyes differ in their chemical properties, but both can be bound to tissue. Lucifer yellow VS is a vinyl sulphone that reacts rapidly with amino and sulphhydryl groups but is extremely stable in water; even in weak base (pH 9.0) its half life is about one week at room temperature. The dye combines rapidly and covalently with proteins and presumably with other tissue constituents containing sulphhydryl or amino groups. Lucifer yellow CH, on the other hand, has a free hydrazido group and reacts with aliphatic aldehydes at room temperature. Aldehyde-containing fixatives bind the CH dye to tissue, presumably through the hydrazido group. Aqueous solutions of this dye appear to be chemically stable for at least several months at room temperature.

So far the CH dye has been used more often than the VS dye, perhaps because the latter is not yet commercially available;

which one is best suited to a particular application can only be determined empirically.

The most frequent application of Lucifer yellow CH has been to reveal the shape of individual neurones¹⁻⁵. It has been used to determine the branching pattern and course of regenerating neurones^{6,7}. Because of the low toxicity of the dye, direct *in vivo* observations on the regeneration of dye-filled neurones may be possible. Lucifer yellow CH has been used in the study of developing systems: in the development of molluscs⁸, for example, and in the exciting work by Goodman and Spitzer on the development of the grasshopper nervous system⁹. I describe here other applications of these fluorescent tracers in the hope that their many potential uses can be rapidly exploited.

Dye-coupling revealed by intracellular injection of Lucifer yellow

Neurones and other cells can be marked with Lucifer yellow CH by injecting the dye through a micropipette using pressure or iontophoresis. The dye spreads quickly throughout the body and processes of the injected cell, but does not cross the cell membrane¹. The low molecular weight (457.2) and intense fluorescence of the dye offer several advantages over other tracers. (1) A cell can be stained in its entirety with a relatively small amount of dye. Illumination sufficient for photomicrography of live cells does not noticeably alter their physiology. (2) In preparations which are sufficiently transparent one can determine whether the distribution of dye found after fixation is the same as that seen in the living state. (3) Most importantly, the dye frequently spreads *in vivo* from the injected cell to certain nearby cells. This movement of dye from cell to cell has been termed dye-coupling¹. In certain cases, dye-coupled cells are known to be electrically coupled to each other. Two examples are horizontal cells in the turtle retina¹ and the septate axon of the crayfish (K. Futamachi and W.S., unpublished). In the embryo of the mollusc *Patella*, however, dye-coupling between macromeres was detected without knowing whether the cells were electrically coupled and was used to study the development of the embryo⁸. Presumably in many (but not necessarily all) instances dye-coupling and electrical coupling have a common basis. In any case, dye-coupling offers a method of recognizing certain functional connections between cells by morphological means.

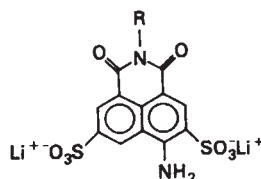


Fig. 1 Structural formula of Lucifer dyes. Both dyes are 3,6-disulphonated 4-aminonaphthalimides. For the lithium salt of Lucifer yellow CH (often referred to simply as Lucifer yellow CH), R is $-\text{NH}-\text{CO}-\text{NH}-\text{NH}_2$; for the lithium salt of Lucifer yellow VS, R is $m\text{-phenyl-SO}_2\text{-CH=CH}_2$. About $\times 24,000,000$.

A pair of neurones called Leydig cells occurs in each segmental ganglion of the leech *Hirudo medicinalis*. These cells are known to be electrically coupled³⁰, and Fig. 2 shows that they are dye-coupled as well. Two neurones in the ganglion contain dye: the Leydig cell that was injected and the contralateral Leydig cell into which dye spread. The transfer of dye presumably occurred at the sites of contact between the arborizations of the two neurones. Unexpectedly, dye also spread from the injected neurone to processes in the ipsilateral axon bundles

leaving the ganglion (Fig. 2b). These are probably axons from Leydig cells in adjacent ganglia, as Kent Keyser has independently demonstrated in greater detail (personal communication). As both adjacent ganglia had been removed before injection, the sites of coupling to the two dye-coupled processes must have been in the remaining ganglion or in the short lengths of attached nerve. Here and in similar situations Lucifer yellow CH is useful for gross localization of the sites of intercellular communication.

The sensitivity of Lucifer yellow CH was compared semiquantitatively with that of two other tracers, Procion yellow and horseradish peroxidase. Horseradish peroxidase is often used for intracellular staining^{18,19} and is detectable by both light and electron microscopy. When 5% solutions of Lucifer yellow CH and horseradish peroxidase were injected by pressure into leech neurones, the two tracers appeared roughly equal in their ability to reveal morphological detail as judged visually with the light microscope (W.S. and K. Muller, unpublished). Horseradish peroxidase, however, cannot be seen in living tissue and is thought not to pass between dye-coupled cells²⁰. Procion yellow, a fluorescent tracer previously used for intracellular injection²¹, has about 1% of the fluorescence intensity of Lucifer yellow and in comparably marked cells reveals much less morphological detail¹.

Backfilling with Lucifer yellow CH

Neurones can also be filled with dye through their processes rather than by injection of dye into their somata. Backfilling, introduced by Iles and Mulloney²², is carried out by simply immersing the cut end of a nerve in a pool of tracer. The tracer moves up the axons of the nerve, either by diffusion or in response to an externally applied electric field, and fills the cell bodies of the neurones with processes in that nerve. This technique has been used with Procion yellow²², with cobalt chloride^{23,24}, and with horseradish peroxidase²⁵. Lucifer yellow CH is at least as suitable for backfilling as these tracers. Moreover, since neither the dye nor the process of backfilling appears to alter physiological properties noticeably, and since the dye can usually be seen *in vivo*, one can first identify a backfilled cell by its fluorescence, then impale it with a micropipette and investigate its electrophysiology.

Figure 3a is a fluorescence photomicrograph of the buccal ganglia of the freshwater planorbid snail *Biomphalaria glabrata*. The preparation was backfilled with the lithium salt of Lucifer yellow CH through the nerve innervating the left salivary gland. The two largest cells containing dye can usually be identified by other criteria such as size, position, and branching pattern. These cells appear to be salivary effector neurones and may be homologous to the cells described by Kater in *Helisoma*²⁶ and Benjamin in *Lymnaea*²⁷. The fluorescent cells were impaled after they had been backfilled, and their physiology appeared normal. In particular, action potentials in the left cell produced one-for-one depolarizing potentials in the salivary cells (Fig. 3b, c), and depolarizing and hyperpolarizing current passed from either cell to the other, suggesting that they were electrically coupled (Fig. 3d, e). These results are similar to those obtained from preparations which had not been backfilled. After fluorescence photomicrography of the ganglia (Fig. 3a), the physiology of both cells was still normal, and they remained electrically coupled (Fig. 3f, g). In this system, therefore, the amount of light needed for fluorescence photomicrography is much less than that which causes photosensitized damage or killing of cells that contain dye. In the method of cell killing devised by Miller and Selverston, the illumination level which killed Lucifer-filled cells in 5 min was 1,000 times the level required for visualization of processes in the neuropil²⁸.

Lucifer dyes as tracers in electron microscopy

Since the Lucifer dyes described here do not contain a heavy atom, they have no useful electron density. Their intense

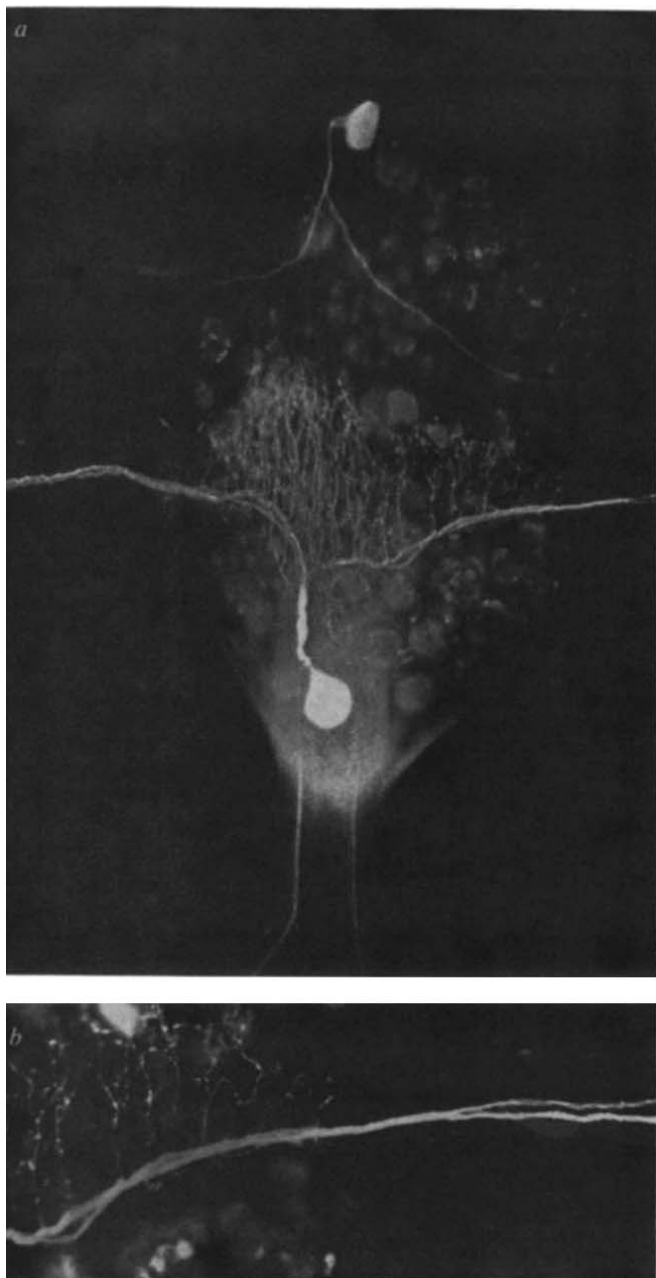


Fig. 2 Leech ganglion containing a Leydig cell injected intracellularly with the lithium salt of Lucifer yellow CH. *a*, The lower cell was pressure-injected with a 5% solution of dye. The tissue was allowed to stand for 90 min at room temperature and was then fixed at 4°C for 12 h in 4% formaldehyde in 0.15 M sodium phosphate buffer, pH 7.4. The ganglion was embedded in glycol methacrylate as previously described¹. The fainter cell body (top) is the contralateral Leydig cell, which is dye-coupled to the injected cell. $\times 107$. *b*, Detail from *a*. The ipsilateral nerve contains two fluorescent processes—one from the injected neurone, the other from a dye-coupled neurone in the adjacent ganglion (see text). $\times 180$.

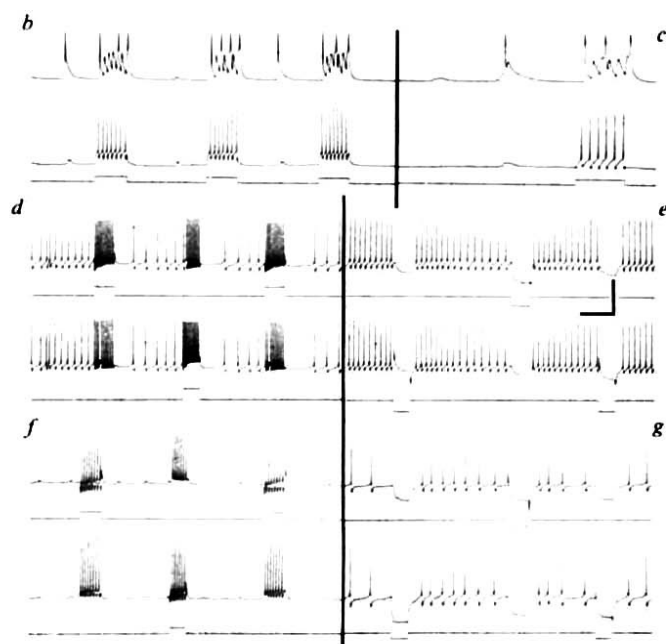


Fig. 3 Live neurones stained by backfilling with the lithium salt of Lucifer yellow CH, and electrical responses recorded after staining. The cut end of the left salivary nerve of a snail (*B. glabrata*) was drawn into a small drop of a 3% solution of dye on a square of polyethylene about 1 mm on each side. For 1 h a potential was imposed so that a positive, constant current of 30 nA flowed from the bath to the drop of dye. The buccal ganglia were rinsed in a Ringer's solution, and the stained cells were identified with fluorescence optics. The large dye-filled cell in the left buccal ganglion was impaled with a microelectrode (*b* and *c*, lower trace). A salivary cell was impaled with a second electrode (*b* and *c*, upper trace). The microelectrode was then withdrawn from the salivary cell and used to impale the large cell on the lateral margin of the right buccal ganglion (*d* and *e*, records for the right cell are on top). The current traces show when current (about 0.25 nA) was injected, using an active bridge circuit (not accurately balanced in all records), first into one cell and then the other. The live preparation, *a*, was then photographed using epifluorescence with a Zeiss Ultraphot. The conditions of photography were: 6.3X Neofluar lens (0.20 numerical aperture), HBO 200 W mercury arc lamp, heat filter and BG-12 excitation filter, FL-500 dichroic splitter, 500 nm barrier filter, 30 s exposure. $\times 66$. After photography, both lateral cells were impaled again, producing the records shown in *f* and *g*. Vertical calibration indicates 50 mV for all records; horizontal calibration indicates 1 s for *c* and 2 s for all other records.

fluorescence, however, allows them to be used as tracers for electron microscopy by an indirect procedure. An ultrathin section mounted on a grid is examined with a fluorescence microscope to identify cells or processes that contain dye, and then the same section is examined with the electron microscope. Figure 4 shows light and electron micrographs of the same areas of a neurone in the right parietal ganglion of the snail *B. glabrata*; this neurone can often be identified by its size and position. To make this type of comparison, tissue is conventionally processed for electron microscopy except that osmication is omitted, since even brief exposure to osmium tetroxide greatly reduces Lucifer fluorescence.

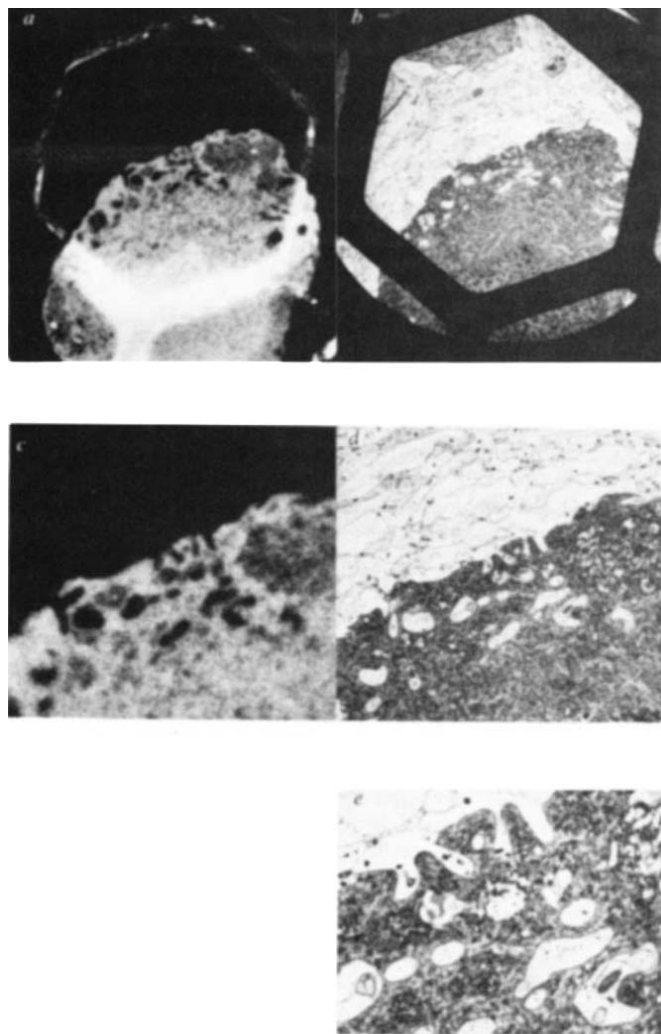


Fig. 4 A cell in the right parietal ganglion of the freshwater snail *Biomphalaria glabrata* was injected with Lucifer yellow CH, fixed for 4 h at 23 °C in a pH 7.4 12.5 mM Na^+/H^+ HEPES buffer containing 2% glutaraldehyde, 1% formaldehyde, and 25 mosM balanced salts, dehydrated in graded alcohols, and transferred to anhydrous acetone at 4 °C. The ganglion was stained for 1 h at 4 °C in 1% hafnium tetrachloride in acetone, then rinsed in acetone and embedded in Spurr's resin⁴². (The fixative and rinse solutions also contained 1% HfCl_4 in this experiment, but not in most.) *a*, Silver section on a hexagonal grid (Polaron) was illuminated through a Zeiss 63X OD lens (0.90 numerical aperture) with an Osram 50 W HBO lamp and photographed with a 15 s exposure on Ektachrome 400 film pushed to ASA 800. $\times 640$. *b*, The same section shown in *a*, photographed with the electron microscope after uranium and lead staining. $\times 640$. *c*, Detail from *a*. $\times 1,500$. *d* and *e*, Detail from *b*. $\times 1,500$ and $\times 3,700$. Photographed at 80 kV.

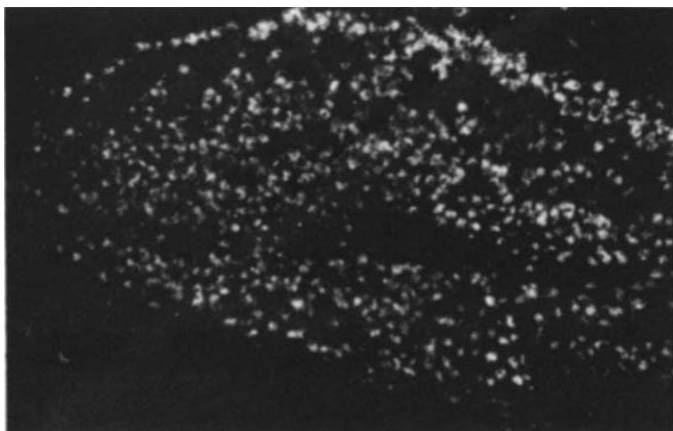


Fig. 5 Isthmo-optic nucleus of chick labelled by retrograde axonal transport of Lucifer yellow VS. The vitreous humour of the contralateral eye was injected with 25 μ l of 5% Lucifer yellow VS; the injection damaged a portion of the retina. After 21 h the chick was anaesthetized and fixed by perfusion with buffered 4% formaldehyde. The other eye was not injected, and its contralateral isthmo-optic nucleus showed almost no fluorescence. $\times 200$.

Post-fixation staining can be omitted entirely, but tissue processed in this way generally lacks sufficient contrast and electron density to give useful images. Adequate contrast is generated by staining tissue *en bloc* in 1% hafnium tetrachloride in anhydrous acetone at 4 $^{\circ}$ C, a procedure introduced by Morris Karnovsky (personal communication). The tissue is then rinsed in acetone and embedded in Spurr's resin. When a thin section (grey or silver) on an ordinary electron microscope grid is illuminated using epifluorescence with an intense arc lamp, the fluorescence of the marked cell is bright enough to be photographed on a fast colour film with a 15-s exposure. The same thin section is then conventionally stained with uranium and lead; the areas that had been found fluorescent are located and photographed with the electron microscope. Surprisingly small areas can be identified with confidence when the light and electron micrographs are compared. In Fig. 4c, d, for example, there are dye-free areas only 500 nm in diameter that are easily recognized in both the light and electron micrographs. The areas from which dye is excluded lie within the perimeter of the cytoplasm of the injected cell and may be penetrating processes of other cells²⁹. Dye-filled processes 500 nm in diameter can usually be found with ease, while 200 nm processes are locatable with greater difficulty.

The necessity of avoiding osmication to preserve fluorescence entails some disadvantages. Membranes either cannot be seen or, more often, appear as thin grey lines (Fig. 4e) instead of the dark black bilayer commonly seen with conventional fixation. The poor visualization of membranes makes the method described here unsuitable for some applications. For example, synapses in the leech central nervous system are difficult to recognize because membranes and associated synaptic specializations lack the contrast they normally have with osmium post-fixation (K. Muller, personal communication).

Since Lucifer yellow CH has no significant electron density of its own, it was possible to test whether either the dye or the process of injection had an effect on the ultrastructure of an injected neurone. The paired Retzius cells of the leech³⁰ were convenient test objects. In each of three segmental ganglia, one Retzius cell was injected with dye and one was not. The tissue was immersed in a cacodylate-buffered fixative²⁰, then processed as described in the legend to Fig. 4. When thin sections

were examined with the electron microscope, both cells were recognizable by their size and position. (The injected cell had previously been identified by its fluorescence.) The ultrastructural morphology of the two cells was virtually the same, suggesting that neither the dye nor the process of injection had caused significant morphological changes (K. Keyser and W.S., unpublished). This experiment illustrates an advantage of an ultrastructural tracer that does not rely on electron density for its detection: the appearance of the marked tissue is not necessarily changed.

Retrograde axonal transport

A useful technique for studies of vertebrate neuroanatomy relies on the phenomenon of retrograde axonal transport, which causes certain exogenous proteins to be carried from axon terminals to the cell body³¹. Kristensson, Olsson, and Sjöstrand

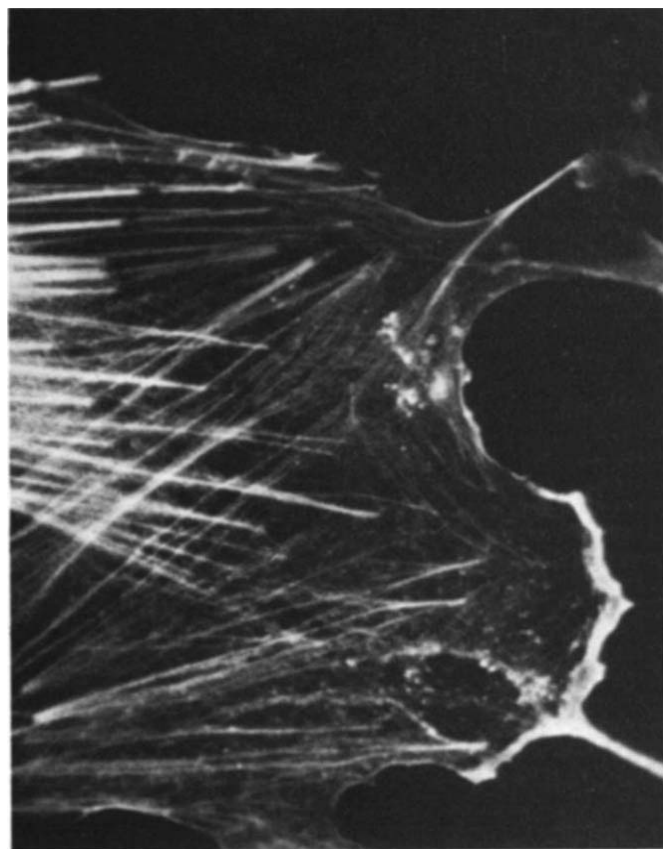


Fig. 6 Indirect immunofluorescence staining of actin filaments with Lucifer-labelled antibodies. Cultured human lung cells, W138, were fixed and treated with rabbit anti-actin antibody, then with Lucifer-labelled goat IgG anti-(rabbit IgG); the procedures were those described by Lazarides⁴³. The Lucifer-labelled antibody was prepared as follows. To 50 mg of Miles-Yeda goat IgG anti-(rabbit IgG) dissolved in 1.8 ml 0.5 M pH 9.0 carbonate buffer was added 0.52 mg Lucifer yellow VS in 1.0 ml carbonate buffer. These weights correspond to a nominal 3 mol of Lucifer per mol of protein, but since Lucifer yellow VS is hygroscopic, less dye was actually present. The mixture was allowed to stand for 3 h at room temperature, then unreacted dye was removed by chromatography with phosphate-buffered saline on Sephadex G-25. The optical densities at 280 nm and 430 nm indicated a dye to protein molar labelling ratio of approximately 2.3. About $\times 500$.

showed in 1971 that horseradish peroxidase appeared in the cell bodies of neurones near whose terminals a solution of horseradish peroxidase had been introduced³². This marker has since been used both to trace pathways in the central nervous system and to study the mechanism of transport itself. The LaVails and others have shown that peroxidase moves within axons at a rate of about 100 mm per day (ref. 33). Some exogenous proteins seem not to be subject to retrograde axonal transport³⁴, but the reasons for this are not understood. Kuypers and his colleagues have recently shown that certain other fluorescent dyes are also subject to retrograde movement^{35,36}, so it is not surprising that Lucifer yellow VS serves as an effective marker for retrograde axonal transport from the chick eye to the cell bodies of neurones which have their axon terminals within the retina³³ (Fig. 5). Perhaps the dye is transported as a protein conjugate formed *in situ*. It is not known whether Lucifer yellow VS will prove useful for tracing connections in other parts of the nervous system.

Recently wheat germ agglutinin labelled covalently with Lucifer yellow VS (see below) has been found to provide good staining of motoneurons in the spinal cord of chick embryo after a small amount (0.5 μ l of 0.5% agglutinin) was injected into leg muscles (M. McPheeters and L. M. Okun, personal communication). After retrograde transport had occurred, it was possible to dissociate the spinal cord and isolate the labelled neurones by means of a fluorescence-activated cell sorter³⁷.

Covalent labelling of macromolecules with Lucifer yellow VS

Lucifer yellow VS labels proteins rapidly and covalently under mild conditions. In 0.1 M NaHCO₃ the reaction is complete in 2 h at room temperature. The conjugates generally have quantum yields between 0.1 and 0.2 (R. Chen and W.S., in preparation), so Lucifer-conjugated antibodies should be suitable for immunofluorescence staining. Figure 6 shows actin filaments of cultured fibroblasts stained with Lucifer-labelled antibodies. Lucifer yellow VS is similar to fluorescein isothiocyanate in its ease of use and fluorescence intensity and has the advantage that its emission peak at 540 nm is considerably to the red of fluorescein's at 515 nm, so that its fluorescence contrasts more strongly with tissue autofluorescence. But since Lucifer has no other clear-cut advantage and since less is known about the chemistry and the stability of the bond that is formed, it is likely that fluorescein isothiocyanate will remain the dye of choice for most immunofluorescence applications. When fluorescence is needed at pHs below 6, however, Lucifer yellow VS may be useful. The fluorescence intensity of Lucifer VS is unchanged from pH 2 to pH 10 (ref. 1), an unusual property for a water-soluble dye. This pH-independence makes the dye suitable for

monitoring pH-induced conformational changes in macromolecules.

Other uses of Lucifer yellow dyes

Among published examples of the possible uses of Lucifer yellow dyes it is worth noting that intracellular staining with Lucifer has been followed by immunocytochemical staining with rhodamine-labelled antibodies³⁸; the goal of this approach is to characterize the immune reactivity as well as the electrophysiology of the same cell. This dye also has been used to follow changes in junctional permeability caused by moderate trans-junctional polarization³⁹. Another interesting application is suggested by experiments in which an individual dye-filled neurone or even a part of a neurone was selectively damaged by means of an intense, focused spot of light²⁸. This phenomenon is sometimes referred to as "cell killing," but as the originators of the technique have pointed out⁴⁰, it is not known whether the affected cells are killed or simply inactivated temporarily. Also, it is not yet known what margin of safety there is for cells that do not contain dye. Finally, there is an interesting recent report that when turtle retina is bathed in a calcium-free Ringer's solution containing Lucifer, the bipolar cells, but not other cell types, take up dye from the medium⁴¹. Uptake is not influenced by light, but is blocked by metabolic inhibitors. It will be of interest to see whether similar phenomena occur in other neuronal systems.

Dye-coupled systems of cells have been discovered through the use of Lucifer yellow CH^{10,11}. It has been used to monitor dye-coupling in regenerating neurones¹²⁻¹⁴, and it greatly facilitated the discovery that an electrical connection between two leech neurones was mediated by a pair of small interneurones¹⁵ and was not direct, as had previously been thought. The dye has been used to obtain information on the position of the micro-electrode tip, either in neurones¹⁶ or, in one case, in giant mitochondria¹⁷. But because of its rapid spread, presumably by diffusion, this dye generally gives only limited information on tip position, and so it may not be the best marker for this purpose.

In addition to the two sulphonated 4-aminonaphthalimides that have been used until now, more than 40 compounds in this group have recently been prepared by a convenient one-step synthesis⁴⁴. These compounds have a wide range of substituents on the imide nitrogen, and most are water-soluble and have an intense yellow-green fluorescence. Because of the ease with which functional groups can be incorporated into sulphonated 4-aminonaphthalimides, these compounds will probably find new applications as biological tracers.

The experiment shown in Fig. 2 was performed at the 1978 Leech Course at Cold Spring Harbor Laboratory. I thank Elias Lazarides for carrying out the immunofluorescence staining and Kent Keyser, Enid Applegate, and Jennifer LaVail for collaboration in performing the experiments described in the legends to Figs 3, 4 and 5 respectively.

- Stewart, W. W. *Cell* **14**, 741-759 (1978).
- Peacock, J. H., Rush, D. F. & Mathers, L. H. *Brain Res.* **169**, 231-246 (1979).
- Shafer, M. R. & Calabrese, R. L. *Cell Tissue Res.* **214**, 137-153 (1981).
- Crawford, A. C. & Fettiplace, R. *J. Physiol., Lond.* **306**, 79-125 (1980).
- Takato, M. & Goldring, S. J. *comp. Neurol.* **186**, 173-188 (1979).
- Murphy, A. D. & Kater, S. B. *Brain Res.* **186**, 251-272 (1980).
- Bulloch, A. G. M. & Kater, S. B. *Science* **212**, 79-81 (1981).
- Laat, S. W. de, Tertoolen, L. G. J., Dorrestijn, A. W. C. & Biggelaar, J. A. M. van den *Nature* **287**, 546-548 (1980).
- Goodman, C. S. & Spitzer, N. C. *Nature* **280**, 208-214 (1979).
- Gutnick, M. J. & Prince, D. A. *Science* **211**, 67-70 (1981).
- Spencer, A. N. & Satterlie, R. A. *J. Neurobiol.* **11**, 13-19 (1980).
- Muller, K. J. & Scott, S. A. *Science* **206**, 87-89 (1979).
- Muller, K. J. & Scott, S. A. *Nature* **283**, 89-90 (1980).
- Scott, S. A. & Muller, K. J. *Dev. Biol.* **80**, 345-363 (1980).
- Muller, K. J. & Scott, S. A. *J. Physiol., Lond.* **311**, 565-583 (1981).
- Fettiplace, R. & Crawford, A. C. *Proc. R. Soc. B203*, 209-218 (1978).
- Bowman, C. & Tedeschi, H. *Science* **209**, 1251-1252 (1980).
- Snow, P. J., Rose, P. K. & Brown, A. G. *Science* **191**, 312-313 (1976).
- Muller, K. J. & McMahan, U. J. *Proc. R. Soc. Lond. B194*, 481-499 (1976).
- Muller, K. J. & Carbonetto, S. J. *comp. Neurol.* **185**, 485-516 (1979).
- Stretton, A. O. W. & Kravitz, E. A. *Science* **162**, 132-134 (1968).
- Iles, J. F. & Mulloney, B. *Brain Res.* **30**, 397-400 (1971).
- Pitman, R. M., Tweedle, C. D. & Cohen, M. J. *Science* **176**, 412-414 (1972).
- Tyrer, N. M. & Bell, E. M. *Brain Res.* **73**, 151-155 (1974).
- Zipser, B. *Brain Res.* **182**, 441-445 (1980).
- Kater, S. B., Murphy, A. D. & Rued, J. R. *J. exp. Biol.* **72**, 91-106 (1978).
- Benjamin, P. R., Rose, R. M., Slade, C. T. & Lacy, M. G. *J. exp. Biol.* **80**, 119-135 (1979).
- Miller, J. P. & Selverston, A. I. *Science* **206**, 702-704 (1979).
- Coggeshall, R. E. & Fawcett, D. W. *J. Neurophysiol.* **27**, 229-289 (1964).
- Nicholls, J. G. & Baylor, D. A. *J. Neurophysiol.* **31**, 740-756 (1968).
- Kristensson, K. *Acta neuropath. (Berl.)* **16**, 293-300 (1970).
- Kristensson, K., Olsson, Y. & Sjöstrand, J. *Brain Res.* **32**, 399-406 (1971).
- LaVail, J. H. in *Methods in Physiological Psychology*, Vol. 2 (ed. Thompson, R. F.) 355-384 (Academic, New York, 1978).
- Hendry, I. A., Stach, R. & Herrup, K. *Brain Res.* **82**, 117-128 (1974).
- Kuypers, H. G. J. M., Catsman-Berrevorts, C. E. & Padt, R. E. *Neurosci. Lett.* **6**, 127-135 (1977).
- Kooy, D. van der, Kuypers, H. G. J. M. & Catsman-Berrevorts, C. E. *Brain Res.* **158**, 189-196 (1978).
- McPheeters, M. & Okun, L. M. *Soc. Neurosci. Abstr.* **6**, Abstr. 247.19 (1980).
- Reaves, T. A. Jr & Hayward, J. N. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6009-6011 (1979).
- Spray, D. C., Harris, A. L. & Bennett, M. V. L. *Science* **204**, 432-434 (1979).
- Selverston, A. I. & Miller, J. P. *J. Neurophysiol.* **44**, 1102-1121 (1980).
- Detwiler, P. B. & Sarthy, P. V. *Neurosci. Lett.* **22**, 227-232 (1981).
- Spurr, A. R. *J. Ultrastruct. Res.* **26**, 31-43 (1969).
- Lazarides, E. *J. Histochem. Cytochem.* **23**, 507-528 (1975).
- Stewart, W. W. *J. Am. chem. Soc.* (in the press).

Active folding in the Algerian earthquake of 10 October 1980

G. C. P. King

Bullard Laboratories, Department of Earth Sciences, Madingley Rise, Madingley Road, Cambridge CB3 0EZ, UK

C. Vita-Finzi

Department of Geography, University College London, Gower Street, London WC1E 6BT, UK

A fold controlling the form of the local topography near El Asnam, Algeria, increased in amplitude at the time of the earthquake of 10 October 1980. The deformation occurred rapidly in an elastic-brittle manner and not in the gradual ductile fashion in which folds are normally thought to develop.

AN earthquake of magnitude 7.3 (M_s) occurred near El Asnam, in north Algeria, at 12.25 GMT on 10 October 1980. Hypocentral locations provided by the US Geological Survey ($1^{\circ}25'E$, $36^{\circ}9'N$) and the International Seismological Centre ($1^{\circ}26'E$, $36^{\circ}11'N$) are broadly consistent with the observed damage distribution¹ but their accuracy cannot be assumed to be better than ± 10 km. Aftershocks recorded by French² and British³ groups define a broader region 50-km long aligned broadly NE-SW with shocks having depths of up to 15 km. Extensive surface faulting occurred within this zone (Fig. 1a). A magnitude of 7.3 suggests a geometric moment^{4,5} of 2×10^9 m³, consistent with a slip area 30-km long and 12-km deep with a mean displacement of 6 m. Further work will constrain the seismic parameters more closely but is unlikely to affect the present arguments.

We examine here some of the more striking features of the surface deformation associated with the earthquake and relate them to subsurface processes. Archaeological and stratigraphical evidence is used to demonstrate that some 30 similar earthquakes could account for the local topography and geological structure.

The central section of a detailed fault map of the area is shown in Fig. 1b; the complete map, which was compiled in conjunction with an aftershock study, will be published elsewhere³. A restricted area is chosen for discussion here for three reasons. First, it displays a well developed thrusting surface break which furnished a reliable measure of horizontal slip direction; and, as

this direction is consistent with that determined in preliminary fault plane solutions, the surface break probably bears a simple relationship to the seismogenic fault at depth. Second, the zone of deformation behind the fault is typical of that observed elsewhere in the area. Third, the channel and fill terraces of the Chelif river cross the section and yield information on recent and late Quaternary deformation.

Compressional faulting

In the region shown in Fig. 1b the thrust forms a clear scarp (Fig. 2) crossing fields and orchards which were level before the earthquake. Figure 3 shows the scarp cutting across one of several lines used for laying irrigation pipes in a potato field. The lines in this field show no lateral offset (excluding some disturbance close to the fault scarp) and must therefore be parallel to the horizontal component of the slip vector. The azimuth of N $140^{\circ}E$ derived from this site was confirmed wherever the fault crossed other linear features such as irrigation channels and embankments. The form of the scarp suggests that its dip lies between 30° and 60° , a value consistent with the preliminary fault plane solution^{2,3}.

Slickensides observed near the southern end of the faulting give a spurious slip direction. This is not surprising as rock mechanics does not predict the formation of slickensides at the surface except in unusual conditions⁶. Brittle failure near the surface should always be tensile (as usually observed) and shear motion near the surface will occur only if it can exploit a very

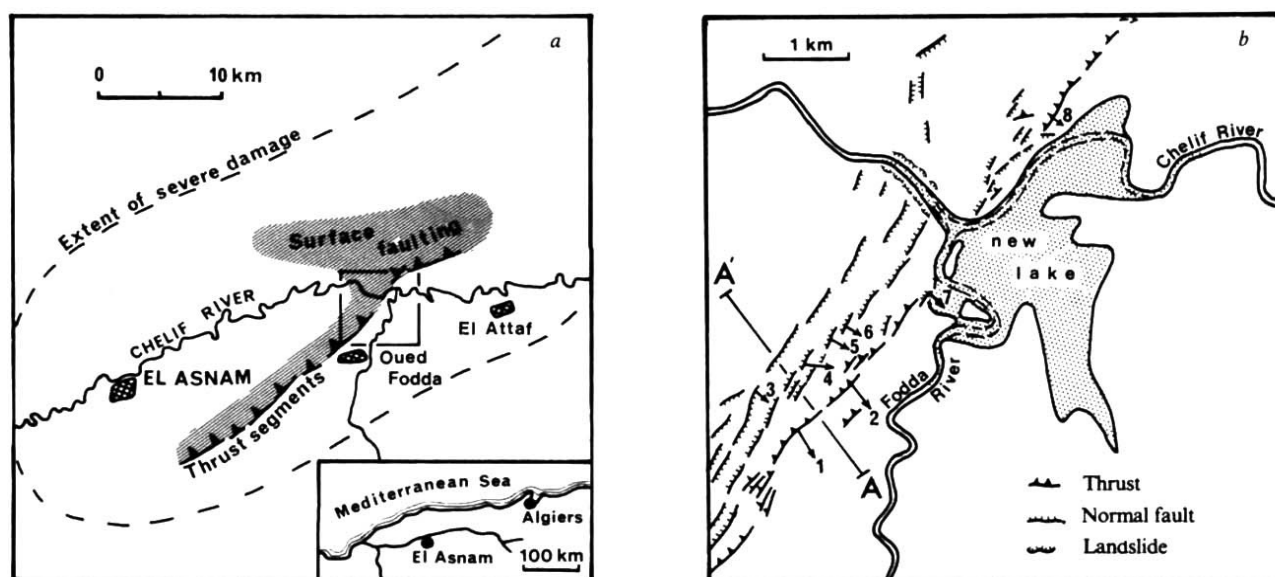


Fig. 1 a, Epicentral region showing approximate extent of severe damage, the principal segments of thrust surface breaks and the region of extensive surface faulting, most of which was tensional. b, Surface breaks near the Chelif river. Slip vector measurements: 1, 1.8 m (vertical component only), azimuth $140^{\circ}E$. 2, 3.2 m (vertical component only), azimuth $135^{\circ}E$. 3, 1.4 m (vertical component only), azimuth $130^{\circ}E$. 4, 1.0 m (total), azimuth $105^{\circ}E$, dip 50° . 5, 0.7 m (total), azimuth $125^{\circ}E$, dip 60° . 6, 0.7 m (total), azimuth $125^{\circ}E$, dip 60° . 7, 1.4 m (vertical component only), azimuth $130^{\circ}E$. 8, 1.2 m (vertical component only), azimuth 130° . The slip vector measurements used in Fig. 2 from section A-A' are not repeated here.



Fig. 2 The thrust surface break north of Oued Fodda (looking north-east). The new fault scarp is 3.2 m high.

weak pre-existing plane. If such a plane exists the slip direction will not necessarily define the broader-scale direction of shortening⁷.

Tensional faulting

Although the reverse faulting is clear and unambiguous in the section we discuss here, the most extensive and striking features of the surface deformation are tensional. In other regions, for example north of El Attaf thrusting becomes difficult to identify but tensional faulting remains obvious.

Figure 4a shows some of the tensional features to be seen when looking south-west from the vicinity of A-A' (Fig. 1b). The faults have produced a graben which seems to be reflected in the existing topography, suggesting previous motion on similar faults. Figure 4b shows a normal fault in a similar environment at the southern end of the fault system. The stratigraphic offset here is several times the displacement of 1 m caused by the present earthquake. Other examples of normal fault reactivation were also found.

Matching of opposite sides of the new tensional cracks^{6,8} showed that, despite wide variation in dip, most of the new slip vectors (including many not shown on Fig. 1b) had horizontal components close to N 140°E.

Two traverses were made across the fault zone to measure slip vectors on fault segments. One of these traverses is indicated by A-A' on Fig. 1b and the information is summarized in Fig. 5d. If no block deformation or rotation occurs between faults then a rigid-body kinematic model can be described using a displacement vector diagram as in Fig. 5c. This suggests that the net effect of hanging wall ruptures is to modify the slip vector (and hence the fault plane) such that its dip increases with depth.

Although a rigid block hypothesis is adequate to describe the surface ruptures alone, it does not explain evidence for rotation and continuous deformation; we therefore reject it. Evidence for rotation and continuous deformation is better explained by a hypothesis of fold formation. This alternative model does not demand a change in fault dip with depth and accounts for all of the observed features.

Evidence for active fold development

The deformation zone is traversed by the Chelif river (Fig. 1a,b). After the earthquake water levels reportedly rose by 5 or 6 m and formed a lake ~5 km² in area. The lake was dammed not by the thrust fault scarp but by a gentle upwarp 1 km further down the valley. Before the earthquake, upwarping had already disturbed the otherwise regular trend of the river gradient. This may be observed by plotting the river profile from the 1/50,000 maps (Fig. 5a) and was noted in 1957 by Boulaine⁹, who also ascribed it to active uplift. No lake is indicated in the 1934 edition of the topographic map (Oued Fodda sheet) but a marsh is shown in the 1890 sheet occupying roughly the same area. The

marsh probably represents the remains of a lake produced in a similar way, which was completely drained by downcutting of the river channel before the 1934 map revision.

The present longitudinal profile of the river is shown in Fig. 5a which also shows the profile of a river terrace that forms a clear, continuous and dateable feature except in the region of maximum uplift. Here the river flows in a gorge¹⁰ and the terrace cannot be traced continuously. Possible terrace fragments exist within the gorge section. Boulaine identified a terrace covered with immature soils at a height of 30 m above the river⁹ (Fig. 5a) but its relation to the terrace outside the gorge cannot be established. Yet even without this information it is clear that the terrace is more uplifted than the present river profile. The uplift must have occurred after the river ceased flowing at the terrace level.

A maximum age for the alluvial deposits of the terrace is provided by Roman sites they have buried in the Chelif basin. The age of the terrace surface is at present less certain but it could be 500 yr (ref. 11). A comparison of the terrace height



Fig. 3 The thrust scarp, here 1.8 m high, crossing a potato field. Photograph was taken along a line used for laying irrigation pipes. The line was not offset by the earthquake.

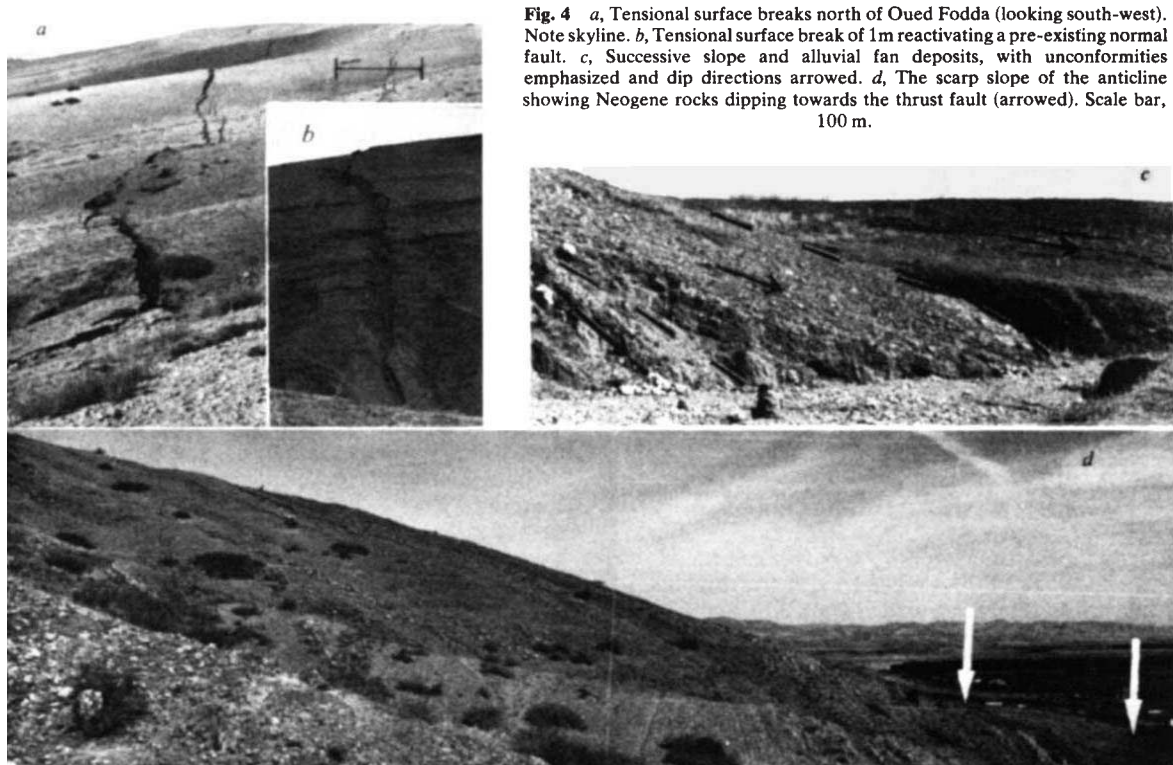


Fig. 4 *a*, Tensional surface breaks north of Oued Fodda (looking south-west). Note skyline. *b*, Tensional surface break of 1m reactivating a pre-existing normal fault. *c*, Successive slope and alluvial fan deposits, with unconformities emphasized and dip directions arrowed. *d*, The scarp slope of the anticline showing Neogene rocks dipping towards the thrust fault (arrowed). Scale bar, 100 m.

with the apparent uplift resulting from the 1980 earthquake suggests that one or possibly two earthquakes of similar magnitude have occurred since the end of deposition.

Figure 5*b* shows a representative structural section across the ridge. The surface breaks we have described occur at the crest and on the south-east flank of an asymmetrical anticline composed principally of late Tertiary (7 Myr) rocks. The deformation displayed by the bedrock (Fig. 5*d*) echoes that of the river profile and of the Chelif terrace. The steeper flank is mantled by scree and alluvial fan material. Ravines have cut through these deposits near the Chelif river to reveal three successive units (Fig. 4*c*), of which the oldest is nearly vertical, the middle dips about 45° and the youngest is in places nearly horizontal. The two oldest deposits contain artefacts that are Lower Palaeolithic, perhaps Acheulean, in type (Fig. 6) and therefore date roughly from the period 1–0.1 Myr ago¹². The youngest deposit contains Mousterian artefacts, which can be ascribed to the period 0.1–0.03 Myr ago¹². Deformation was therefore already in progress between these two time spans. The sequence closely resembles that of the Gafsa area in Tunisia, where conglomerates and sands containing late Acheulean artefacts with dips ranging up to 80° are overlain by alluvial deposits containing Mousterian and later industries. It is no coincidence that the Gafsa structure should also be a 'faulted fold'¹³.

The close relationship between the tilted gravels, the thrust and the anticline north of Oued Fodda suggests that they are co-genetic. If we accept that the deformation of the Chelif terrace indicates one event and a recurrence interval of 500 yr all the topography shown in Fig. 4*d* could have been formed in 15,000 yr by 30 events each with an uplift of 5 m. A similar rate has been demonstrated for anticlinal uplift in the coastal Zagros¹⁴. The long chronology favoured by some archaeologists would, of course, imply longer recurrence intervals. In due course radiometric dating will remove the uncertainty.

Earthquake faulting and surface deformation

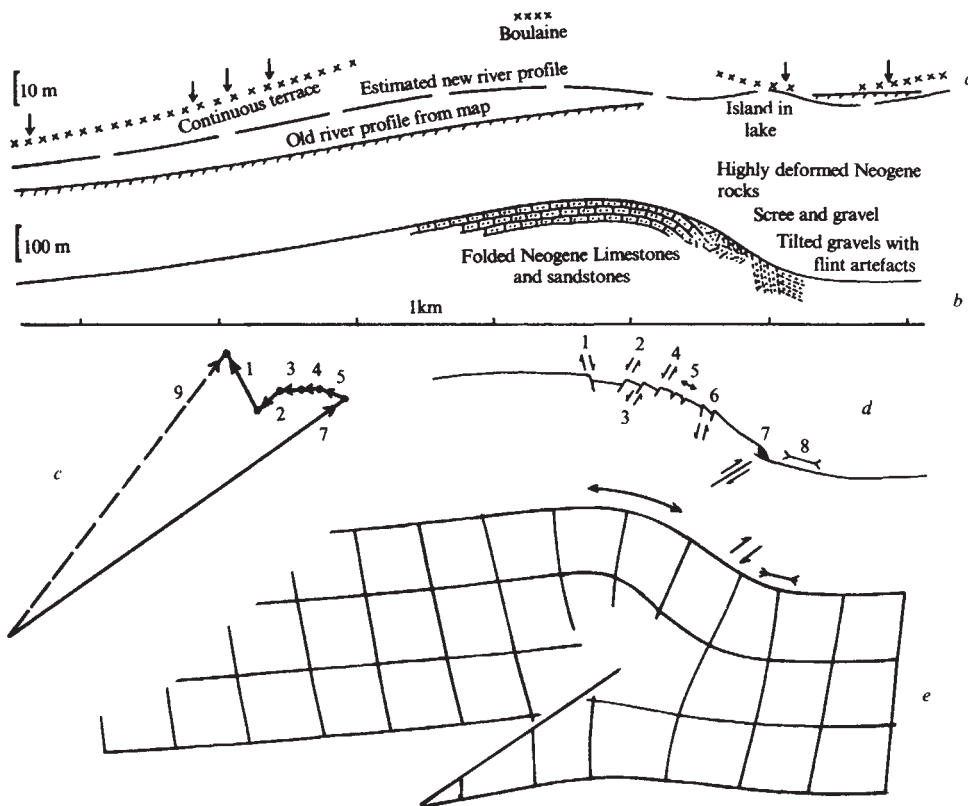
Earthquakes result from failure in rock in elastic–brittle conditions. Localized rupture at the hypocentre extends catastrophically as the rupture front expands over the fault plane until it reaches a region where the progressively increasing stress intensification resulting from increasing fault area is none the

less insufficient to fracture more rock^{15–17}. The general nature of such regions remains enigmatic but certain conditions must prevail as the ruptured front of a crustal earthquake propagates downwards or towards the surface (see Fig. 7).

In regions both near the surface and at depth the rupture enters rock that is relatively stress-free before the earthquake, and fracture is driven into these zones only as a consequence of crack-tip stress. We emphasize that, so far as the rupture processes are concerned, the medium behaves in an elastic–brittle fashion and it is only over long time scales that creep relieves stress. Near the surface, stress is slowly relieved by chemical processes assisted by water flow in cracked or porous rock. These processes are suppressed at depth by confining pressure; at even greater depths thermally activated creep processes relieve stress in metamorphic conditions¹⁸. The depths that bound the elastic–brittle and elastic–brittle–ductile regions are shown in Fig. 7 as 5 and 20 km respectively. These depths are, of course, arbitrary and depend on such diverse factors as rock type, grain size, groundwater flow, thermal gradient and the recurrence period of earthquakes. The behaviour is also modified by complex fault geometry and the presence of highly incompetent layers such as salt. But, in so far as continental earthquakes rarely have hypocentres outside the range of 5–20 km and the surface ruptures of large earthquakes broadly reflect the source parameters determined from seismic observations, the model in Fig. 7 is adequate.

An implication of the model is that the rapid deformation at the time of an earthquake is elastic–brittle in nature. The ductility relieves stress created by the earthquake over a longer time scale and hence 'freezes in' the elastic deformation field produced at the time of the main shock. Following the earthquake, the relaxation of stress in the elastic–brittle–ductile zones will tend to reload the elastic–brittle zone. This reloading provides an additional or an alternative explanation for after shocks to that proposed by Das and Scholz¹⁶ who consider that stress corrosion in a brittle environment permits crack extension which leads to failure; essentially a microscopic process. The process described here will produce similar aftershock time histories but is a bulk process and depends on creep distant from the crack tip. In either case the release of strain in the after-shocks is small compared with the main shock and puts an upper limit on strain change associated with post-seismic creep. Thus

Fig. 5 Representative sections showing faulting and deformation near the Chelif river. *a*, Old river profile from the topographic map, and the new river profile deduced partly from the form of the new lake. The post-Roman (Chelif) river terrace is indicated; arrows show where the height was calibrated with respect to the river profile by levelling. The terrace is a clear continuous feature between the arrowed positions upstream and downstream of the gorge. *b*, Topographical and geological section through the anticline showing the relation between the tilted gravels (as scree and alluvial fans) and the folded Neogene rocks. *c*, Vector diagram showing the effect of the observed tensional surface features on the slip vector at depth assuming a rigid, non-rotating, block model. The vectors correspond to motion on features shown in *d*. 1, 0.9 m, dip 60°, azimuth 100°; 2, 0.3 m dip 45°; 3, 0.3 m, dip 30°, azimuth 140°; 4, 0.5 m, dip 30°, azimuth 145°; 5, 0.2 m over many small tension cracks; 6, small bedding thrusts with negligible offsets in this particular section; 7, main thrust estimated to dip at 50°; 8, region of compression without faulting; irrigation channels in this region were telescoped by between 1 and 2 m over a distance between 200 and 300 m; 9, slip vector at depth. *e*, Deformation around a buried thrust fault with a dip of 35° and dimensionless fault parameters¹⁹ of $d = 1.5$, $D = 1.25$, $L = 10$ and slip of 1.15. Note that *a* and *b* have vertical exaggeration.



an elastic model approximates both the displacement at the time of the earthquake and the strain field following post-seismic stress relaxation. Modelling of the effect of ductility, which may produce long wavelength (but small strain) displacement fields will be discussed elsewhere.

Figure 5e shows the elastic deformation above a buried thrust fault. In this model the fault does not break the surface but the deformation field scarcely differs if the fault reaches the surface with diminished slip amplitude. The model is calculated using analytical finite integral expressions derived by Mansinha and Smylie¹⁹ for the displacement anywhere in a half space due to a buried dislocation surface on which displacement is specified. Figure 5e may be interpreted as representative of the deformation of a single earthquake in which strain has been exaggerated to produce a clear figure, or as an unexaggerated view of the cumulative effects of many similar earthquakes.

Assuming that the fractures represent only a small release of the total strain produced in surface materials, the position and nature of the fractures (Fig. 5d) are well explained by the model

in Fig. 5e. The only fractures poorly described by the model are those marked 6 in Fig. 5d and these had small displacement and were related to steeply dipping beds. This explanation of the surface features, based on a model of continuous deformation, contrasts with that offered earlier using a vector diagram (Fig. 5c), which assumes rigid unrotated blocks and requires the fault plane to steepen with depth. Although continuous deformation models in which fault dip steepens with depth would probably fit our observations, the data do not demand this refinement. The simple continuous deformation model also explains the form of the uplift of the river profile and terrace uplift (Fig. 5a). It also describes the deformation of the Neogene rocks and the present-day morphology (Fig. 5b).

Conclusions

Perhaps the most surprising aspect of the Algerian earthquake was the predominance of tensional surface faulting associated with a compressional event. This effect has been attributed to landslide motion or to changes in the dip of the main thrust plane with depth. The deformation model we present produces both extensional and compressional strain near the surface at the time of the earthquake; but, as surface materials fail more readily in tension than in compression, widespread tensional features are to be expected. The scale of the normal faulting at El Asnam is substantial and has produced major features in Neogene rocks. Thus normal faulting cannot be taken to be a fossil indicator of regional tension unless it is known to extend into the elastic-brittle zone.

The relation between structure, morphology and the location of surface breaks in the recent earthquake was clear and greatly assisted the task of fault mapping. The relation between topography and some of the surface features associated has been dismissed by many field workers as the product of large scale landslides. This view does not wholly contradict our interpretation. The elastic-ductile surface zone must retain sufficient shear strength to support the topography (or the mountains would rapidly fall down²⁰). The stresses that exist before the earthquake modulate the stress associated with the earthquake and thus, to some extent, determine where fault features are observed. In several places, tensional surface breaks increased

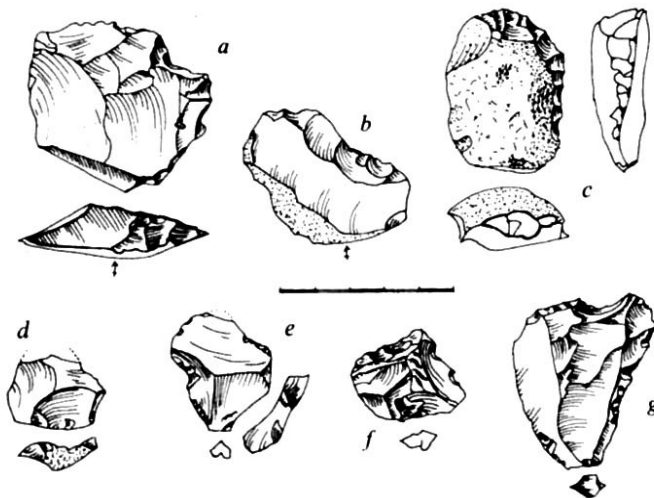


Fig. 6 Artefacts from the tilted gravels; *a*, *b* and *c* from the oldest unit; *d*, *e* and *f* from the intermediate unit; *g* from the youngest (ref. 11) unit. The scale is in centimetres.

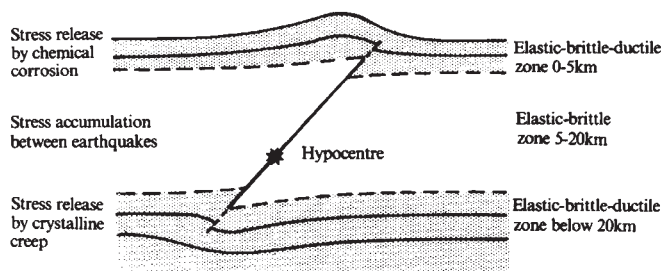


Fig. 7 The stress environment of a shallow continental earthquake.

in slip amplitude near the tops of hills and diminished in the valleys while the opposite was true of compressional surface breaks.

Although the rocks folded in this earthquake are mainly Neogene in age, the present evidence suggests that the observed folding is very much more recent. All of the folds in the region are unlikely to be this young. It is plausible that deformation has moved southwards with time and that the present fold is the frontal fold of an assemblage of serial folds²¹ (a view to be discussed more fully elsewhere), in which case the migration of fold activity should be regarded as the effect of serial faulting. In terms of the Jackson model of orogenic development²², this suggests that deformation in a closing sedimentary basin starts at the centre and spreads outwards.

Received 24 February; accepted 6 May 1981.

1. Ambraseys, N. *Geol. J.* (in the press).
2. Ouyed, M. *et al. Nature* **292**, 26–31 (1981).
3. Yielding, G. *et al.* (in preparation).
4. Kanamori, H. & Anderson, D. *Bull. seism. Soc. Am.* **65**, 1073–1095 (1975).
5. King, G. C. P. *Phil. Trans. R. Soc. A* **288**, 197–212 (1978).
6. King, G. C. P., Soufleris, C. & Berberian, M. *Disasters* (in the press).
7. Bott, M. H. P. *Geol. Mag.* **96**, 109–117 (1959).
8. Mercier, J. L., Mouyaris, N., Simeakis, K., Kondoyannis, Th. & Angelidhis, C. *Nature* **278**, 45–48 (1979).
9. Boulaine, J. *Étude des Sols des Plaines du Chélib* (Service des Études Scientifiques, Birmandreis, 1957).
10. Anderson, R. van W. *Mem. geol. Soc. Am.* **4**, 1–450 (1936).
11. Vita-Finzi, C. *Man* **2**, 205–215 (1967).
12. Isaac, G. L. in *Calibration of Hominoid Evolution* (eds Bishop, W. W. & Miller, J. A.) 381–430 (Scottish Academic, Edinburgh, 1972).

Without suggesting that all folding is as intimately related to faulting as in the Algerian example, one is tempted by the simplicity of the proposed mechanism to regard it as widely applicable. Faults at depths formerly in the elastic-brittle region have been exhumed by erosion in sufficient numbers to account for a substantial proportion of surface folding.

Unlike the conventional view of fold formation as a plastic instability in a homogeneous stress field²³ or as a gradual process in incompetent rock above a competent faulted basement^{24–26}, the observed folding occurs rapidly in conditions of inhomogeneous stress above and as a direct consequence of the properties of the elastic-brittle layer. The model also indicates fold formation beneath the brittle layer and, in so far as the upper and lower layers are arbitrary and time-dependent, within the brittle layer.

We thank Nick Ambraseys, Armando Cisternas, James Jackson, Mustapha Meghraoui, Harsh Sinvhal, Robert Wood and Graham Yielding for assistance. Financial support was provided by the NERC and the Royal Society. The Algerian Government and M. Benaissa as their representative were most helpful. We thank B. Trovato for helpful discussions. This is Cambridge University Department of Earth Sciences contribution no. 150.

Note added in proof: S. Wisnousky has drawn our attention to work related to ours being conducted at Tokyo University by Y. Ikeda and Y. Nobuyuki but at present published only in Japanese.

13. Castany, G. *Geol. Rdsch.* **43**, 196–203 (1955).
14. Vita-Finzi, C. *Nature* **278**, 632–634 (1979).
15. Aki, K. & Richards, P. *Quantitative Seismology* (Freeman, San Francisco, 1980).
16. Das, S. & Scholz, C. H. *J. geophys. Res.* (in the press).
17. Madariaga, R. in *Mécanismes et Prévision des Séismes* (ed. Allègre, C. J.) 125–134 (CNRS, Paris, 1980).
18. Ashby, M. F. & Verrall, R. A. *Phil. Trans. R. Soc. A* **288**, 59–95 (1977).
19. Mansinha L. & Smylie, D. E. *Bull. seism. Soc. Am.* **61**, 1433–1440 (1971).
20. Jeffreys, H. *The Earth*, 4th edn (Cambridge University Press, Cambridge, 1959).
21. Shearman, D. J. *Geogr. J.* **142**, 393–404 (1976).
22. Jackson, J. A. *Nature* **283**, 343–346 (1980).
23. Ramsay, J. G. *Folding and Fracturing of Rocks* (McGraw-Hill, New York, 1967).
24. Stearns, D. W. *23rd A. Field Conf. Guidebook*, 125–144 (Wyoming Geological Association, Laramie, 1971).
25. Matthews, V. (ed) *Mem. geol. Soc. Am.* **151** (1978).
26. Smithson, S. B., Brewer, J., Kaufman, S., Oliver, J. & Hurich, C. *Geology* **6**, 648–652 (1978).

Seismotectonics of the El Asnam earthquake

M. Ouyed*, M. Meghraoui†, A. Cisternas‡, A. Deschamps‡, J. Dorel‡, J. Frechet§, R. Gaulon‡, D. Hatzfeld§ & H. Philip¶

*Centre National d'Astronomie, d'Astrophysique et de Géophysique, Algeria, and Laboratoire de Géophysique Interne et Tectonophysique, ERA No. 603 IRIGM, BP No. 53 X, 38042 Grenoble Cedex, France

†Centre de Recherches et d'Applications en Géosciences, Algeria, and Laboratoire de Dynamique de la Lithosphère, Université de Paris VII, 4, place Jussieu, 75230 Paris Cedex 05, France

‡Laboratoire d'Étude Géophysique des Structures Profondes, LA No. 195, Institut de Physique du Globe de Paris, Université Pierre-et-Marie Curie, 4, place Jussieu, 75230 Paris Cedex 05, France

§Laboratoire de Géophysique Interne et Tectonophysique, IRIGM, Université Scientifique et Médicale de Grenoble, BP No. 53 X, 38041 Grenoble Cedex, France

¶Laboratoire de Géologie Structurale, Université des Sciences et Techniques du Languedoc, Place E. Bataillon, 34060 Montpellier, France

The mechanism of the El Asnam earthquake of 10 October 1980 ($M_s = 7.2$), the strongest recorded in northwestern Africa is compatible with a convergence between the African and European plates. The thrust is also consistent with field observations and with long period data from the global network. Aftershocks cluster around the fault trace, with a maximum depth of 12 km. Maximum vertical displacement is ~6 m and there is some left lateral motion. The fault trace of the 1954 earthquake at Beni-Rached was also reactivated.

THE destructive earthquake in the region of El Asnam (Algeria) on 10 October 1980 (12.25 UTC) was of magnitude $M_s = 7.2$ according to the Europeo-Mediterranean Seismological Center (CSEM) and $M_s = 7.3$ according to the US Geological Survey. Previous earthquakes have occurred in this region on 9 September 1954 with magnitude 6.7 and epicentre at Beni-Rached, and on 7 September 1934 with

epicentre at El Abadia (Carnot) 5 km north of El Attaf ($m_b = 5.0$).

The event was the largest recorded in northwestern Africa, and because of its significance to the area geodynamics, a team of French and Algerian seismologists and geologists began recording the aftershocks and mapping surface breaks within 48 h. Some of their results are reported here.

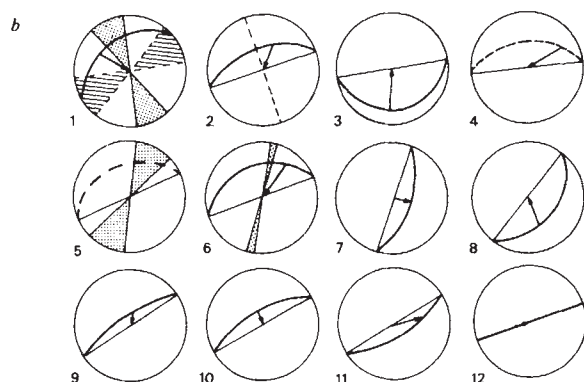
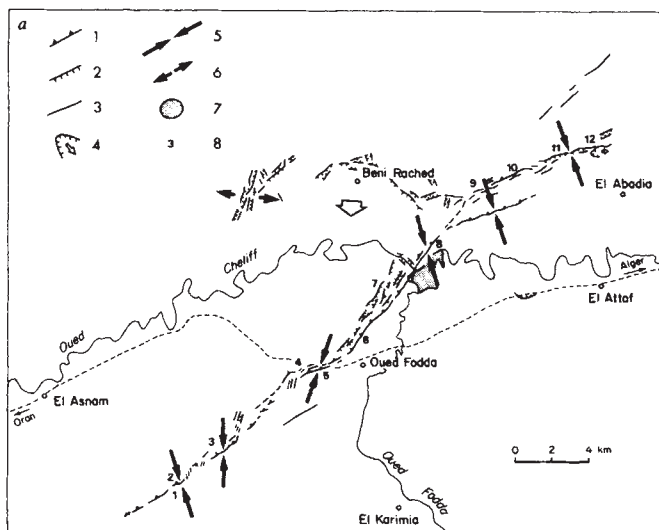


Fig. 3 *a*, Structural map of the epicentral region. 1, Thrust; 2, normal fault; 3, vertical cracks; 4, landslides; 5, direction of compression; 6, direction of extension; 7, flood; 8, site of slip measurements. *b*, Displacement vectors and striations. Stippling, extension cracks; hatching, pressure ridges. Lower hemisphere Schmidt projection shows the fault trace and striation or displacement vectors (4.5.6.).

during the Quaternary before entering the canyon due to successive inundations. Perturbations of the flow of the Chelif river reported⁴ after the 1954 earthquake can possibly be attributed to the same cause. Detailed observations of the Quaternary sediments under the lake⁵ should enable the recurrence record of large earthquakes along the fault to be studied.

Groundwater subjected to a sudden pressure increase was ejected to the surface leaving sand blars characteristic of large earthquakes. Many natural sources were interrupted (near the fault) and others were reactivated (away from the fault).

Main shock

Relocation was used with respect to a well determined reference shock (one recorded by our local network and by the global network (8 November 1980, at 7h 54min, $m_b = 5.3$)) to obtain more precise epicentres for the main shock and the largest aftershock: values of $36^{\circ}11.1'N$, $1^{\circ}22.8'E$ for the main shock, and $36^{\circ}12.5'N$, $1^{\circ}32.0'E$ for the largest aftershock (10 October 1980 at 15h 39min; $m_b = 6.0$) were obtained with standard error of 1.6 and 1.5 km respectively (Fig. 1). These differ by less than 8 km from values obtained by the USGS and the CSEM. Because our method fails to provide good depth control, we regard the vertical distribution of the aftershocks which give a maximum of 12 km for the depth of the main shock and of the largest aftershock as an important constraint. The data on the focal mechanism of the main shock, from the long period stations of the global network are shown in Fig. 2a. The fault plane can be determined accurately, but the auxiliary plane is

uncertain. Our solution has no strike-slip ($\phi = 230^{\circ}$, $\delta = 52^{\circ}$, $\lambda = 90^{\circ}$), but field observations suggest that a small component of left lateral motion is present, at least in the southern part of the fault zone. Field observations also allow the seismic moment to be estimated. If we assume an average displacement of 4 m, a fault length of 40 km and a fault width of 15 km from the aftershocks distribution $\mu = 3.10^{11}$ dyn cm⁻², we obtain a seismic moment $M_0 = 7.2 \times 10^{26}$ dyn cm. A similar seismic moment of $M_0 = 4.1 \times 10^{26}$ dyn cm was also obtained by modelling long period P-wave recorded at various stations. Figure 2b compares observed and theoretical seismograms using a simple point source in an homogeneous medium and the radiation pattern given by Fig. 2a. The source function is trapezoidal in shape with a total duration of 12 s. On some of the stations (PRE, GRM, ARO, BTG, LPA) a secondary arrival is observed some 3 or 4 s after the first one. This arrival is not seen at SHK, MAT, KEV and JKF and may be due to fault propagation. The arrival times of the reflected phases led us to work with a focal depth of 3.5 km; the position of the relocation, however, meant that this was too shallow: A more complex time function of the source leads to a greater depth: the introduction of a sedimentary layer above the source ($v_p = 4.5$ km s⁻¹) has little effect on the waveforms.

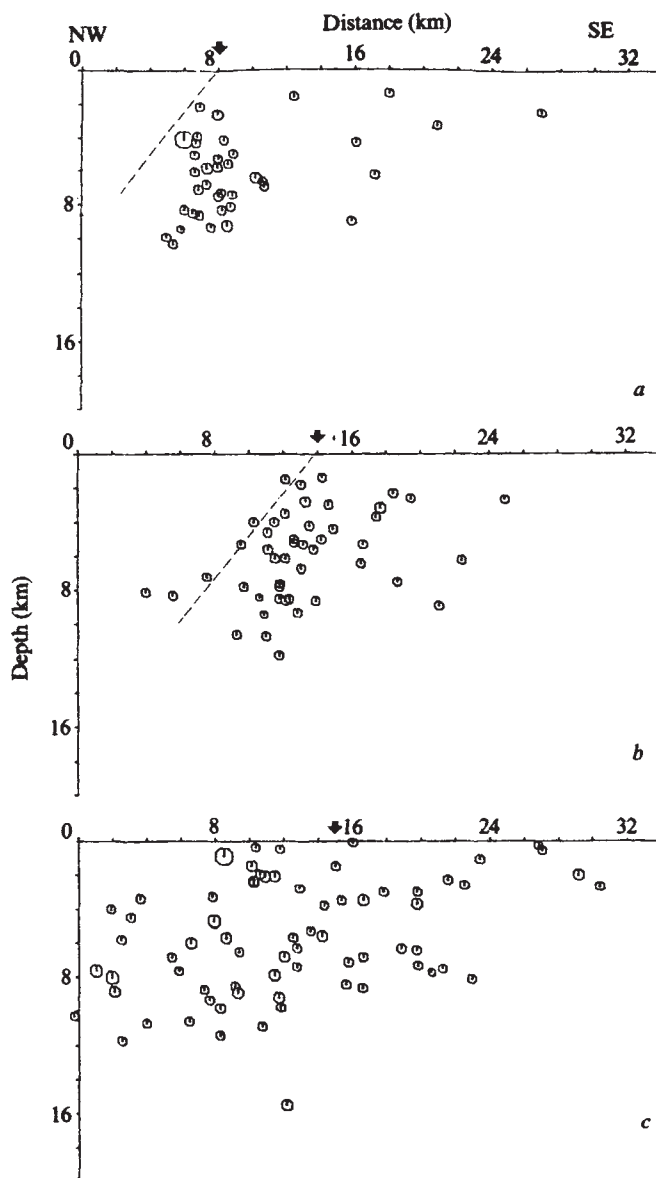


Fig. 4 Vertical distribution of the hypocentres of aftershocks at three different cross-sections (see Fig. 1). The complexity of the distribution increases from south-west (*a*) to the north-east (*c*). Dashed lines show the fault plane from teleseismic data.

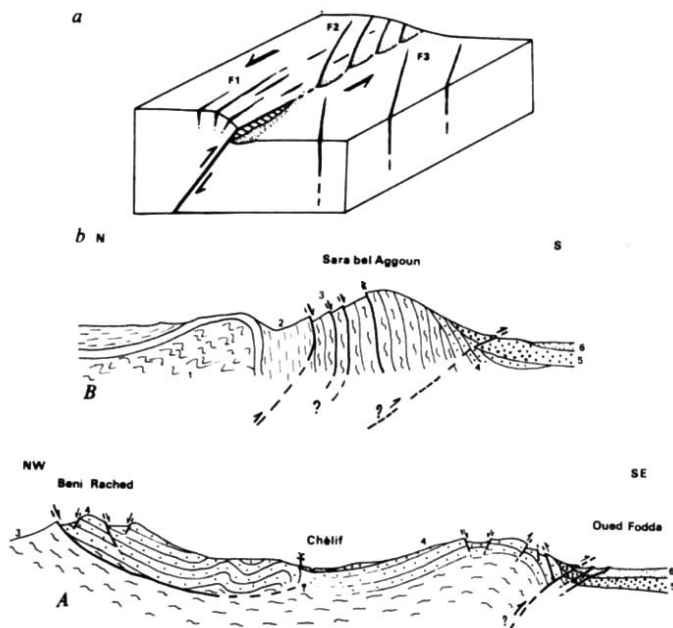


Fig. 5 *a*, Block diagram showing the structures at southern end of the fault. *F*₁, Flexure cracks; *F*₂, left lateral *en échelon* cracks; *F*₃, extension cracks parallel to pressure axis. *b*, Schematic cross-sections. *A*, NW-SE section through Beni-Rached: 1, flysch; 2, medium Miocene marls; 3, higher Miocene marls; 4, Pliocene sandstone; 5, conglomerates; 6, recent Quaternary. *B*, N-S section through the anticline north-west of El Abadia (Fig. 3, site 10).

A stress drop of 70 bar can be estimated from the field observations using the procedure of Kanamori and Anderson⁶.

Aftershock activity

A network of 14 seismic stations (11 portable Sprengnether MEQ-800 paper recorders and three 3-component analogue magnetic tape event recorders) were installed around the fault and operated for more than a month starting on 12 October. For clear records in conditions of strong aftershock activity, a low

gain was selected. A drum speed of 12 cm min⁻¹ was chosen and to obtain precise readings time marks were activated every second. Epicentres of 150 aftershocks within the period between 12 to 22 October 1980 (see Fig. 3) were determined using the HYPO 71 program with a layer of 3 km of 4.5 km s⁻¹ over an half space of 6 km s⁻¹. Most of the aftershocks correspond to local magnitude (calculated from duration) smaller than 3. There is a clear difference between the *b*-values at the north-east and those at the south-west side of the fault trace. For example, the station at El Tnine, (NE) recorded ~500 events per day, whereas that at Ouled Ben Abdel Kader (Massena) (SW) had only 50 events per day, the difference being smaller for the larger events located near these stations. There is clear indication of migration of seismicity at this stage of the analysis. A striking feature of the seismicity pattern is the gap in activity between the epicentres of the main shock and the city of Oued Fodda. This gap may be due to the fact that, after the main shock, most of the stress was released. Note that there is almost no seismicity around the trace of the curved fault at Beni-Rached which was activated during the 1954 earthquake. Overall aftershocks are concentrated towards the ends of the fault trace, but there is also a cluster towards the centre, north of the city of Oued Fodda.

Figure 4 shows cross-sections drawn at three places along the main fault and aftershocks contained within 11 km on each side of the cross-section projected on a vertical plane. Almost all the aftershocks have depths <12 km, with a standard error of 2–3 km. Sections *a* at the south-west and *b* at the centre of the fault suggest a steeper fault plane than that given by the focal plane solution of the main shock. This slope is more pronounced in section *a*, indicating either a discontinuity or a twisting of the fault surface. Seismic activity is concentrated on the southeastern block. On the other hand the cross-section *c* shows a very diffuse pattern of seismicity. This area has the most complex surface breaks. The fact that a fan of surface cracks is observed suggests a multiple branching of the fault at depth at the end where the tearing stopped. This complexity might also explain the abundance of microearthquakes there compared with the smaller number of events recorded at the southwestern end.

Many records provided clear first readings and an attempt was made to construct mechanisms for the different clusters of aftershocks. Those located to the southwestern side of the fault

Fig. 6 *a*, The 1.30-m left lateral slip measured on a ploughed at Zebadja near the centre of fault. *b*, Southern segment of fault. Typical pressure ridge showing the uplift of the northwestern block (to the left), collapse due to gravity. Extension cracks follow the compression



trace, show a coherent composite mechanism that agrees rather well with the NW–SE compression and associated thrust of the main shock. But the data from the centre and the north-east give incoherent composite solutions. The only inference that we may draw at present is that the fault complexity seen at the surface also occurs at depth.

Tectonic structures

A large area was affected by surface fracturing (Fig. 3). The fault is relatively linear on the southern segment (N050) but it becomes wider and more complex towards the north as it approaches the hills on the right bank of the Chelif. This complex fault is superimposed on the complex geological structure of this area: the southwestern Miocene monocline gradually becomes a strongly folded and faulted structure towards the north-east (see cross-section, Fig. 5). The combined effect of rugged topography and the strong dip of layers of different strength in the folded area, may generate landslides of considerable extension and it is not easy to separate structures due to faulting from mass displacements produced by gravity. This is the case at Beni-Rached (see Fig. 3).

(1) Southern segment of the fault: the fault trace seems to be continuous in this region but a detailed analysis shows a succession of segments connected by *en échelon* cracks. There were several notable common features observed in this region.

A topographic flexure, some metres in size, with extension cracks parallel to the axis of the fold (see Fig. 5b, A) commonly occurred with the main break at the inflection point of this flexure. A striated fault plane is sometimes present. The striations indicate thrusting with an occasional left lateral motion component. At the sites with soft topography (small inclination of the fault plane near the surface) cracks (Fig. 3), some metres to tens of metres long are disposed *en échelon* indicating a left lateral displacement (see Fig. 5a). In all cases, outside the flexured area, we may see extension cracks that seem to be parallel to the pressure axis (Fig. 5a). In many places, where the surface soil is thick and unconsolidated (cultivated fields) the fault is recognized by a succession of pressure ridges (10–150 cm high) with the axis parallel to overall fault strike. The pressure ridges are shifted along extension cracks *en échelon* corresponding to transform faults. Similar surface features, which are very common along surface breaks during large shocks, were observed for the Tabas-e-Golshan earthquake⁷, and the Paríahuana earthquake⁸ (Peru) (Fig. 6b).

(2) Northern segment: near the city of Oued Fodda the fault changes direction from N050 to N070 for ~3 km, and returns to N050 for ~10 km. At this point the fault spreads into several branches, some of which again have a N070 direction. Northwest of Oued Fodda the faulted zone is superimposed on an important topographic step, corresponding to an anticline affecting a Mio-Plio-Quaternary terrain. This anticline zone becomes N080 north of El Adabia. Here the faulting becomes more diversified and there are a large number of breaks with the aspect of normal faults. These normal faults are localized on the anticline, while below the relief there is a region of compression folds and thrusts with important displacements (up to 2.5 m) north of Oued Fodda (see Fig. 5b, A). On the other hand, motion along the segment at N070, between the lake formed by the Chelif river and El Abadia, is modest (15–30 cm of horizontal displacement).

Normal faults within this region show different characteristics. In many cases they are the result of the extension of the surface layers of the fold (Fig. 3, site 7) or they may also correspond to the gravitational collapse of the upper block of the thrust. Those observed north of El Abadia, may result from slip along bedding planes with amplitudes of up to 3 m. This case occurs frequently in this zone, and steeply dipping beds seem to guide the deformation. Also, a fault with a 'normal' aspect on the surface may become a thrust at depth. This could be the case in Fig. 3 at points 10, 11 and 12, and Fig. 5b, A. Analogous cases of normal faults that show an aspect of reverse faults at the surface, have been demonstrated⁹ for the Thessaloniki earthquake.

More complex deformation mechanisms are observed in the folded region at the north, the whole anticline structure being affected. The left lateral component of the displacement at the south may be absorbed here. This hypothesis agrees with the progressive increase of the vertical slip towards the north-east. (3) Dislocations at Beni-Rached and Sidi Djilali: a continuous system of normal faults with large offsets extends for several kilometres near Beni-Rached in an arcuate shape near the interface between the thick limestone series of the Miocene marls and the Pliocene sandstones. This geometry suggests a large gravity slide. Several workers have described important water jets at the base of the relief at the level of the Chelif valley, together with slow motions of the soil for several days after the main earthquakes. Note that these were the same structures that were activated during the 1954 earthquake¹⁰. The cross-section in Fig. 5b, A shows that the folds in the Pliocene layers on the right bank of the Chelif valley may be explained by successive repetitions of the large landslides at the base of the Pliocene sandstones during the Quaternary, with the folds at the Chelif level accounting for the extension in Beni-Rached. However, the complexity and size of the deformation mechanism means that a tectonic origin for these features is also possible. A detailed study of the aftershocks may throw some light on the nature of the tectonics at depth.

At Sidi Djilali (5-km west of Beni-Rached), the interpretation of the structures becomes more difficult. Some of the structures may be the continuation of those present at Beni-Rached, but others, corresponding to a graben parallel to the line of steepest slope have a tectonic origin. The direction of the grabens is roughly parallel to the compression axis as measured at the level of the main fault. The same interpretation may be given to certain graben to the east of Beni-Rached near the intersection with the main fault (Fig. 3). Also, a single focal mechanism corresponding to the only aftershock (8 km in depth) located at Sidi Djilali (Fig. 1), although constrained by the small number of stations, is compatible with a WNW extension of the graben.

Slip amplitude

A topographic survey conducted by the Algerian SNTF revealed important movements across the fault along the rail track from Algiers to Oran. The mean slope was 2% before the earthquake and is now 2% over 300 m. The levelling 3 days after the main shock shows that the changes are discontinuous, the largest being 6 m. The vertical displacements decrease to the South of the fault zone and disappear near the RN 19 highway (Fig. 3, site 1). To the north, vertical displacements are often larger than 1 m (2.5 m north of Oued Fodda) and reach 4 m north of El Abadia.

The amount of horizontal displacement increases from the south to the centre of the fault. It is zero at the level of RN 19 and amounts to ~1.30 m of left lateral slip at the centre (Zebadja) (Fig. 6a). It may reach 2.70 m to the north of Oued Fodda. It was measured by the offset of linear structures traversing the fault.

A measure of ground shortening was possible using a water duct orthogonal to the fault trace north of Oued Fodda. We obtained a total shortening of 2.2 m over 300 m—following the relative displacement of the duct with respect to the soil would give a separate confirmation of this observation—this figure provides a lower limit on shortening across the fault as the aqueduct did not cross the entire zone.

Conclusions

We have shown that, except for Beni-Rached and Sidi Djilali where gravity has a role, the nature of observed dislocations is consistent with a thrust with some left lateral slip in the South. The focal mechanism determined from long period data agrees with surface tectonics and aftershock distribution. Seismic moment calculations determined independently from local seismotectonic evidence and from long period P-data also give similar values.

The complexity of the fault increases from the south-west to the north-east, as indicated by surface evidence, aftershock distribution, the difficulty to obtain composite mechanisms, and *b*-value variations. The main fault, some 40 km in length which has been active during the Quaternary, is associated with folding of the Miocene, Pliocene and Quaternary formations, and is composed of segments of two different directions N050 and N070; flexure on the northwestern side of the main rupture produces a set of normal faults.

The 1980 earthquake, in contrast with the 1954 earthquake, shows a remarkable concordance between surface tectonics and seismic focal mechanisms giving a stronger constraint for plate motions. In particular, the shortening of ~2.50 m gives an upper limit to the relative motion between Africa and Europe of

the order of 0.8 cm yr⁻¹, as there has not been such a large event in the past 300 yr.

We thank Paul Tapponier for helpful discussions and Geoffrey King for improving the presentation of this paper. This study was financed by the Institut National d'Astronomie et Géophysique (France). We thank the Ministère de l'Enseignement supérieur et de la Recherche Scientifique (Algeria), le Centre National d'Astronomie d'Astrophysique et de Géophysique (Algeria) and the Centre de Recherches et d'applications en Géosciences (Algeria) for logistic support, and the French Embassy at Algiers for assistance. The Service de Physique du Globe, Rabat, Maroc, collaborated with instruments and personnel.

Received 9 February; accepted 16 April 1981.

1. Bousquet, J. C. & Philip, H. *Sedimentary Basins of Mediterranean Margins, URBINA* (in the press).
2. McKenzie, D. P. *Geophys. J. R. astr. Soc.* **30**, 109–185 (1972).
3. Tapponier, P. *Bull. Soc. géol. Fr.* **7**, 437–460 (1977).
4. Thevenin, J. *Terre Eaux, Alger* **24**, 14–23 (1955).
5. Sieh, K. E. *J. geophys. Res.* **83**, 3907–3939 (1978).

6. Kanamori, H. & Anderson, D. L. *Bull. seism. Soc. Am.* **65**, 1073–1095 (1975).
7. Berberian, M. *Bull. seism. Soc. Am.* **69**, 1861–1887 (1979).
8. Philip, H. Megard, F. *Tectonophysics* **38**, 259–278 (1977).
9. Mercier, J. L., Mouyaris, N., Simeakis, K., Kondoyannis, Th. & Anghelidhis, C. *Nature* **278**, 45–48 (1979).
10. Rothé, J. P. *La Nature* **3237**, 1–9 (1955).
11. Groupe de recherche Néotectonique de l'Arc de Gibraltar. *Bull. Soc. géol. Fr.* **7**, 575–614 (1977).

A human *onc* gene homologous to the transforming gene (*v-sis*) of simian sarcoma virus

Riccardo Dalla Favera, Edward P. Gelmann,
Robert C. Gallo & Flossie Wong-Staal

Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205, USA

The human genome contains a single and constant locus (c-sis) related to the transforming gene (v-sis) of simian sarcoma virus. The isolation of a recombinant phage containing the entire human c-sis gene has allowed the study of its genomic organization: c-sis extends over a region of ~12 kilobases (kb) which includes 1.2 kb of v-sis-related sequences interrupted by four intervening sequences.

RETROVIRUSES have been identified as the aetiological agents of naturally occurring tumours in several animal species (see ref. 1 for reviews). Furthermore, recent evidence indicates that they can sometimes be isolated from certain human T-cell leukaemias and lymphomas (see refs 2, 3 and review in ref. 4). Some of these agents rapidly induce tumours when inoculated into animals and can transform cells *in vitro*⁵. The genomes of these viruses contain sequences, called viral *onc* genes, which are directly responsible for transformation both *in vitro* and *in vivo*⁵. Substantial evidence indicates that these *onc* genes originated from normal cellular genes by recombination between a parent non-transforming virus and host cellular DNA⁵. Molecular hybridization experiments have shown that the cellular genes that gave rise to viral transforming genes are conserved throughout evolution⁵ and that their rate of evolutionary divergence parallels that of globin sequences⁶. This evolutionary conservation suggests that cellular *onc* genes may code for important functions in cell growth or tissue differentiation.

As these normal cellular genes are homologous to viral transforming genes, their potential role in tumorigenesis is of great interest. Several examples suggest that transformation could, in some cases, be due to an increased level of *onc* gene expression. In the case of the Rous sarcoma virus, the protein product of the viral transforming gene (*src*) is indistinguishable from that of its cellular homologue (*sar*)^{7–9}. The protein (pp60^{src}) is present at low levels in uninfected cells and its amount is elevated 100-fold in transformed cells^{7–9}. Another cellular *onc* gene, *c-myc*, related to the avian myelocytomatosis virus (MC29) is also implicated in neoplastic transformation. B-cell lymphoma

induction in chickens by the non-acute leukosis virus (RAV-2) is sometimes associated with 'downstream' promotion of *c-myc*^{10,11}.

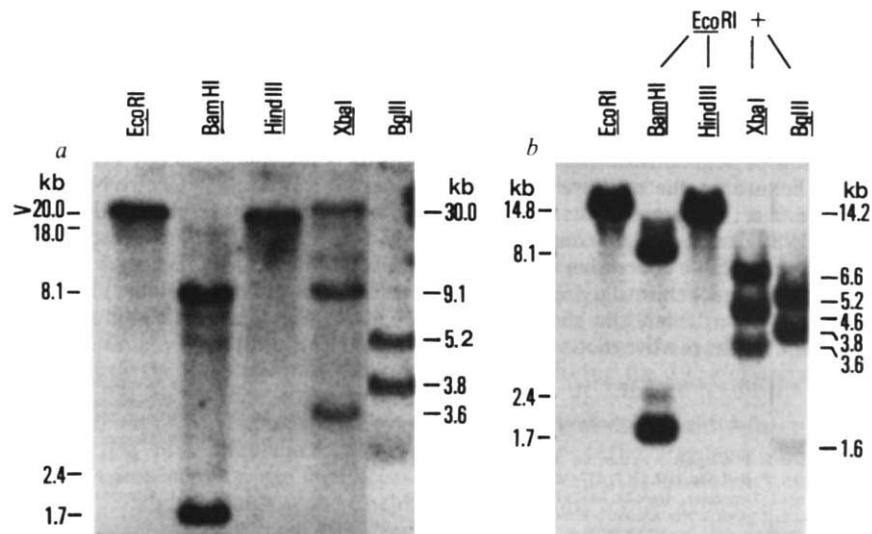
These observations underline the importance of studying the structure and function of human *onc* gene. Reports of preliminary experiments have confirmed the presence of some of these cellular *onc* genes in the human genome^{6,12–14}. Here we report the detection, molecular cloning and genomic organization of a newly described human *onc* gene (*c-sis*) related to the transforming gene (*v-sis*) of simian sarcoma virus (SSV).

The human genome contains *v-sis*-related sequences (*c-sis*)

SSV is a defective transforming virus isolated from the fibrosarcoma of a woolly monkey^{15,16}. The genomes of both SSV and of its helper, simian sarcoma-associated virus (SSAV), were recently cloned in our laboratory¹⁷. The recombinant phages containing SSV (λ-C60) and SSAV (λ-B11) were characterized and compared with each other. The SSV genome was found to have a 1.2-kilobase (kb) insert, not shared by SSAV, which represents the viral *onc* gene (*v-sis*)¹⁷. Molecular hybridization experiments suggest that *v-sis* is a unique viral *onc* gene originating from woolly monkey DNA (F.W.-S. *et al.*, unpublished data).

To investigate whether the human genome contains sequences related to *v-sis*, we analysed human DNA by restriction enzyme digestion and Southern blotting¹⁸. Both SSV and SSAV were subcloned in the plasmid vector pBR322 (ref. 19) and the resultant recombinant plasmids (pC60 and pB11,

Fig. 1 Restriction pattern of *c-sis* in total human DNA (a) and in clone λ -L33 (b). a, DNA from the human cell line HL60 (ref. 38) was prepared by cell lysis, proteinase K digestion, phenol extraction and ethanol precipitation³⁹; 20 μ g were digested with 40 units of the appropriate restriction endonuclease in standard conditions recommended by the supplier. Fragments were separated by electrophoresis on a 0.8% agarose gel. DNA was denatured and transferred to nitrocellulose as described by Southern¹⁸ and hybridization was performed using dextran sulphate⁴⁰. The probe used was 2×10^6 c.p.m. of nick-translated¹⁸ SSV DNA (clone pC60). pC60 is a recombinant plasmid derived from λ -C60, the recombinant λ phage in which the entire SSV genome was originally cloned¹⁷. For subcloning into plasmid, the λ -C60 insert was excised by *Eco*RI digestion, isolated on agarose gel and ligated into *Eco*RI-cleaved and bacterial alkaline phosphatase-treated pBR322 (ref. 41). Subclone selection was performed by colony hybridization to 32 P-labelled SSV/SSAV cDNA⁴². b, A partial *AluI*-*Hae*III human DNA library (from T. Maniatis)⁴¹ was screened using 32 P-labelled pC60 as a probe: 2.5×10^5 plaques were screened and one positive signal was obtained. The corresponding phage (λ -L33) was plaque-purified three times and grown in large scale²¹. λ -L33 DNA was purified and 2 μ g analysed as detailed in a. Molecular weights are in kilobases (kb).



respectively) were used to probe genomic blots of human DNA. Twelve different samples of human DNA from normal tissues, cell lines, leukaemic leukocytes and solid tumour tissues were digested with several restriction enzymes, run on agarose gel and hybridized in duplicate to pC60 and pB11 DNAs labelled with 32 P by nick translation²⁰. In all cases the same set of bands was detected by pC60 (an example is shown in Fig. 1a) but no hybridization was obtained using pB11 (not shown). The fact that *v-sis* is the only region of pC60 not present in pB11 indicates that human DNA contains a cellular homologue (*c-sis*) of the SSV transforming gene. To confirm that the hybridization was specific for *v-sis*, different plasmid subclones of pC60 were used as probes. Only those known to contain *v-sis* (sequences detected some or all of the bands identified with pC60 (data not shown). Analogous results were obtained with DNAs from several vertebrate species, confirming the evolutionary conservation of *c-sis*¹⁴. In all human DNAs tested *c-sis* is contained in a 20–25-kb fragment delimited by *Eco*RI and *Hind*III restriction sites. Within this region of DNA there are internal cleavage sites for several other restriction enzymes (*Bam*HI, *Xba*I and *Bgl*II, Fig. 1a) and these are also conserved among different DNA samples. These data suggest that *c-sis* is a single-copy gene with a constant locus in the human genome.

Isolation of a recombinant phage containing human *c-sis*

A clone of the *c-sis* gene was isolated from a recombinant phage library of human DNA²¹. 32 P-labelled pC60 was used as a probe to screen 2.5×10^5 phage, corresponding to about one genome. A single recombinant phage (λ -L33) was isolated by the Benton and Davis plaque hybridization technique²². After *Eco*RI digestion to release the insert from the Charon 4A (Ch 4A) vector arms, the DNA was analysed by Southern blotting with the same enzymes used in the analysis of the genomic DNAs. The results are shown in Fig. 1b. Digestion with *Eco*RI showed a single 14.8-kb band. Ethidium bromide staining of the gel (not shown) revealed three fluorescent bands: the same 14.8-kb insert and the two vector arms (20 and 10.5 kb), indicating that λ -L33 insert has no *Eco*RI internal cuts. Thus, the size of the human DNA insert in λ -L33 is 14.8 kb. Digestion with *Hind*III yielded a band of 14.1 kb; an additional band of 0.7 kb visible in the gel did not hybridize to pC60. Therefore, comparison with Fig. 1a suggests that most of the *Eco*RI-*Eco*RI and *Hind*III-*Hind*III regions are present in the 14.8-kb λ -L33 insert. Within this region all the *v-sis*-related fragments generated by *Bam*HI

and *Bgl*II are the same size as genomic DNA fragments generated by the same enzymes. The two *Xba*I sites of *c-sis* in genomic DNA are also present in λ -L33 as shown by the presence of three bands in λ -L33. One band (3.6 kb) in the genomic blot is identical in size to a band in λ -L33 (Fig. 1b) and the other two, corresponding to ~40 kb of genomic DNA, are partially represented in the 14.8-kb λ -L33 insert.

In all the enzymes tested (*Xho*I, *Sst*I and *Pst*I; not shown) there were no bands visible in the genomic blot that did not have equivalent bands in the λ -L33 blot. These data suggest that the entire *c-sis* is included in the clone. This was further confirmed by the facts that: (1) 32 P-labelled λ -L33 detected all the *v-sis*-specific fragments in a Southern blot filter containing a restriction digest of pC60 (data not show); and (2) heteroduplex analysis between λ -L33 and λ -C60 showed that the region of homology between the two clones is ~1.2 kb, the estimated size of *v-sis* (see Figs 4, 5). The availability of a clone containing the entire *c-sis* allowed us to study its genomic organization.

Genomic organization of *c-sis*

Two techniques were used to locate the regions in λ -L33 homologous to the 1.2-kb *v-sis* sequence. *v-sis*-related sequences in λ -L33 were mapped by restriction enzyme analysis

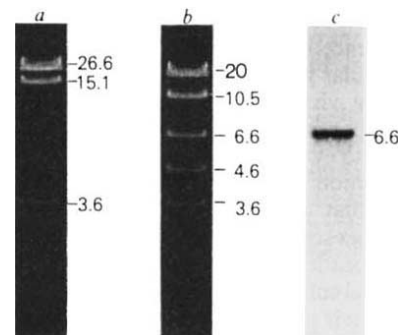


Fig. 2 5'→3' orientation of *c-sis* in λ -L33. a, *Xba*I-digested λ -L33 DNA visualized by ethidium bromide staining. b, *Eco*RI+*Xba*I-digested λ -L33 DNA (ethidium bromide staining); 20- and 10.5-kb bands represent the left and right arms of Ch 4A, respectively. c, Hybridization of DNA from b to a probe containing the 5'-specific region of *v-sis* (plasmid pBG3). pBG3 was constructed by ligation of a *Bgl*II fragment of pC60 (ref. 17), containing 0.35 kb of SSAV-related sequences and the first 5' 0.3 kb of *v-sis*, into *Bam*HI-cleaved pBR322.

Fig. 3 *v-sis* homologous regions are fragmented in *c-sis*. λ -L33 DNA (100 ng) was digested simultaneously with *Eco*RI and *Xba*I restriction endonuclease. Fragments were run on a 1% agarose preparative gel. Bands at 6.6, 4.6 and 3.6 kb, corresponding to the human DNA insert, were purified from agarose by the NaI-glass powder binding method²⁴ and 100 ng from each fragment were digested separately, run on a 1.4% agarose gel and transferred to duplicate nitrocellulose filters. One set of filters was hybridized to ³²P-pC60 DNA, the second to ³²P-labelled λ -L33 DNA. *a*, Hybridization to pC60; *b*, reconstructed restriction map of λ -L33 derived from hybridization of each *Xba*I fragment to ³²P-labelled λ -L33 DNA (blots not shown); *c*, brackets indicate the position of fragments (shown in *a*) which hybridize to pC60.

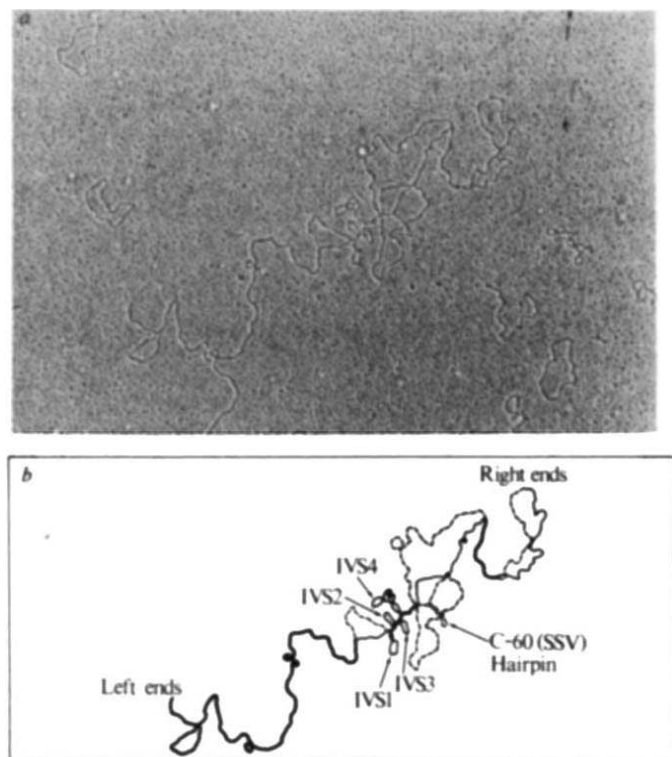
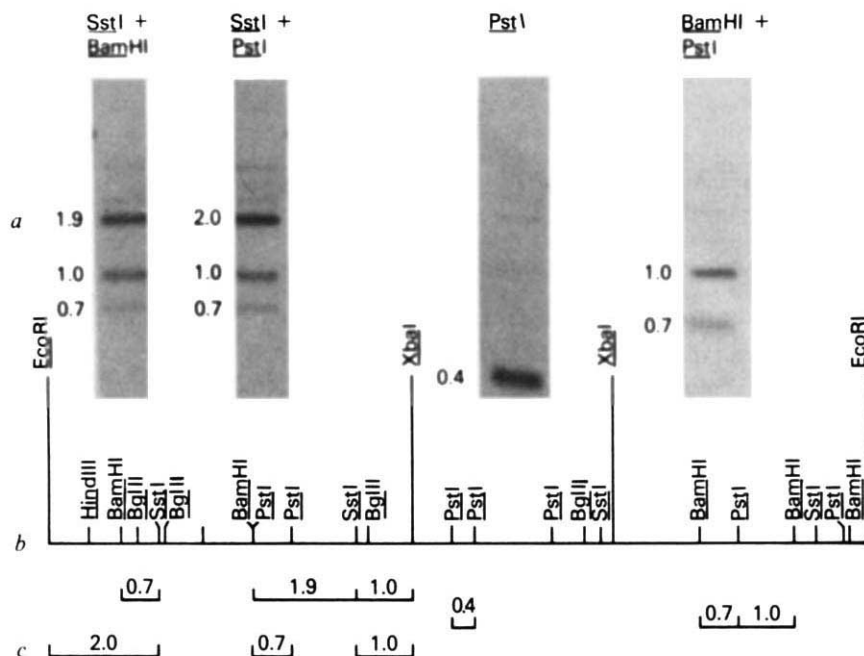


Fig. 4 Heteroduplex analysis of *c-sis* and *v-sis*. *a*, Electron micrograph of heteroduplex structure formed by annealing λ -L33 DNA and λ -C60 DNA. Heteroduplex formation was carried out in 50% formamide and 25 °C after alkali denaturation of caesium-banded phage particles⁴³. Heteroduplex spreading used a hyperphase of 50% formamide and a hypophase of 20% formamide⁴³. Magnification $\times 14,000$. *b*, An interpretive drawing of the heteroduplex structure. Intervening sequences are designated IVS 1–4 in the 5' $\rightarrow$ 3' direction of *c-sis* and SSV DNA.

and heteroduplex molecules were formed between λ -L33 and the recombinant phage containing the SSV genome (λ -C60).

Restriction enzyme analysis: The orientation of *c-sis* in λ -L33 was determined using the endonuclease *Xba*I which does not cut either arm of Ch 4A (ref. 23). Digestion of λ -L33 with *Xba*I yielded three fragments of 26.6, 15.1 and 3.6 kb (Fig. 2a). When the human DNA insert was released from the vector arms by *Eco*RI digestion, the 26.6- and 15.1-kb fragments were reduced

to 6.6 and 4.6 kb, respectively, which suggests that they are linked respectively to the left (20 kb) and right (10.5 kb) arm of Ch 4A (Fig. 2b). The 3.6-kb band is conserved in both digestions; thus this fragment is not linked to vector arms and is located centrally in the λ -L33 insert. To determine the 5' $\rightarrow$ 3' orientation with respect to the *sis* coding sequences, *Eco*RI-*Xba*I-digested λ -L33 DNA was hybridized to a probe specific for the 5' region of *v-sis*. Only the 6.6-kb fragment hybridized to this probe (Fig. 2c). Thus the data suggest that the orientation of *Xba*I fragments in λ -L33 is: (Ch 4A left arm)-5'-6.6 kb-3.6 kb-4.6 kb-3'-(Ch 4A right arm). For simplicity we mapped each *Xba*I fragment separately after isolation on a preparative agarose gel²⁴. Restriction digests of each fragment were used to make duplicate Southern blots to hybridize to ³²P-labelled λ -L33 or p-C60 DNA. λ -L33 bands (not shown) allowed the construction of the map shown in Fig. 3b.

The *v-sis*-related bands are shown in Fig. 3a for each of the three fragments. These bands represent sequences of homology in five noncontiguous regions (bracketed in Fig. 3c). Fragments detected by λ -L33 but not by pC60 represent DNA consisting only of sequences unrelated to *v-sis*. In the case of the internal *Xba*I fragment these must be due to intervening sequences interrupting *v-sis*-related regions. Both intervening sequences (that is, between hybridizing regions) and flanking cellular sequences are present in the 5' and 3' *Xba*I fragments of *c-sis*.

The restriction enzyme analysis suggests the presence of five *v-sis* homologous regions interrupted by four intervening sequences. To analyse further this structure, electron microscopic studies were performed.

Heteroduplex analysis: Human *c-sis*, cloned in Ch 4A, and the 6.3-kb SSV genome, cloned in Ch 21A (Ch 21A) (ref. 17), were compared by heteroduplex formation. A typical molecule is shown in Fig. 4. The left arm of Ch 21A (C60) is 21.2 kb, of which 19.8 kb from the end are identical to the entire left arm of Ch 4A (L33) (ref. 23). Two portions of the right arms are the same²³ and form an interrupted duplex. The hairpin loop structure in λ -C60, formed by the self annealing of a direct and inverted long terminal repeat (LTR)¹⁷, provides a convenient marker for locating *v-sis* at the 5' side of the hairpin. Here a region of hybridization (~ 1.3 kb) between λ -C60 and λ -L33 can be seen. This region contains four deletion loops which represent sequences in the λ -L33 insert nonhomologous to *v-sis*. As they are between regions of homology they are most likely the four intervening sequences identified in the restriction enzyme map. Between the left arm area of the vectors and the region of homology there is a substitution loop formed by the

5'-flanking cellular sequences of λ -L33 (which do not hybridize to the SSV helper sequences 5' of *v-sis*) and the remaining 1.4 kb of the Ch 21A left arm not included in the Ch 4A left arm. A large substitution loop 3' of *v-sis* includes SSAV-related sequences of C60 and the Ch 21A right arm facing λ -L33 flanking cellular sequences and the Ch 4A right arm. Measurements and orientation of the left substitution loop, duplex regions and the deletion loops (Fig. 5) are consistent with the map derived from the restriction enzyme analysis. A general scheme of the genomic arrangement of *c-sis* is shown in Fig. 5.

Repeated sequences in *c-sis* introns

In heteroduplex experiments, λ -L33 single-stranded molecules showed various secondary structures. Furthermore, when λ -L33 was used as a probe in genomic blot experiments with human DNA, a hybridization smear was visible (data not shown). These observations prompted us to check the presence of repeated sequences in λ -L33, specifically, the *Alu* family of interspersed repeats because most repeated sequences in the human genome belong to this family²⁵. Accounting for 3–5% of human DNA, the *Alu* family consists of several 300-base-paired fragments bearing a single *AluI* site²⁵. A recombinant plasmid (BLUR 8) containing a 300-base pair *Alu* repeat insert²⁵ was used as a probe to ascertain the presence of these repeats in λ -L33. Several regions of hybridization were seen in restriction digests of λ -L33 DNA (data not shown). To locate the repeats more precisely, each *XbaI* fragment was mapped as previously described. Results are shown in Fig. 6b. Segments containing *Alu* repeats are located in three separate regions included in two different intervening sequences. Their approximate positions are also shown in Fig. 5.

Discussion

Vertebrate genomes contain nucleotide sequences homologous to the transforming genes of oncogenic retroviruses^{5,6,12–14,26,27}. Our study extends this observation to the cellular homologue of *v-sis*, a newly characterized viral transforming gene¹⁷, most likely originated from woolly monkey DNA (F.W.-S. *et al.*, unpublished data). We report elsewhere¹⁴ that *c-sis* is conserved among vertebrate species and here we describe the characterization of the *c-sis* locus in the human genome. Our data suggest that *v-sis* homologous sequences are present in a single and constant locus in human DNA. This locus showed no polymorphism in our analysis of 12 different DNA samples. *c-sis* consists of a DNA sequence 12 kb long where 1.2–1.3 kb of *v-sis* homologous sequences are interrupted by four intervening

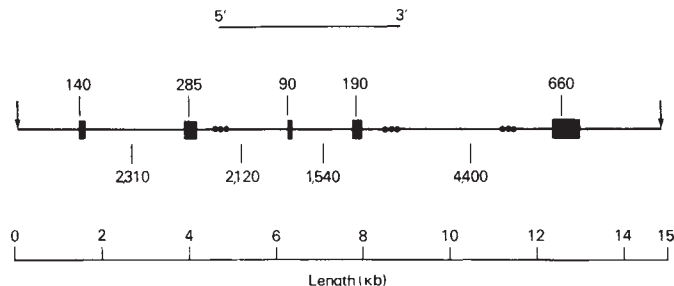


Fig. 5 A schematic representation of the organization of *c-sis* sequences in λ -L33. The lengths of the *v-sis* homologous regions (black boxes) and intervening sequences (solid lines) are mean values derived from measurements of at least six heteroduplex structures. Standard deviations range from $\pm 5\%$ for the larger to $\pm 15\%$ for the smaller measurements. Charon 4A DNA sequences are not shown here; arrows indicate the ends of the cloned genomic fragment. ●●● Indicate the approximate position of repeated sequences of the human *Alu* family (see text and Fig. 5). kb, Kilobases.

sequences. The presence of intervening sequences has been shown in all but one case by studies of cellular *onc* genes from the species of origin of the respective retroviruses. Examples are the cellular *onc* genes of Abelson leukaemia virus in mice, of Harvey sarcoma virus in rats, Rous sarcoma virus and avian erythroblastosis virus in chickens, and feline sarcoma virus in cats^{13,26}. Although *v-sis* is not originated from human DNA, the general structure of *c-sis* has been preserved throughout evolution, which suggests that it may represent a functional gene in man. If *c-sis* and *v-sis* code for a similar protein as in the Rous sarcoma system⁹, the intervening sequences would represent noncoding regions (introns) which would be excised during RNA processing.

A separate observation reported here is the presence of repeated sequences related to the *Alu* family of repeats in *c-sis* introns. The significance of this finding is unknown. Members of this family of repeats are estimated to be present $\sim 300,000$ times throughout the human genome²⁵. They have been found at the 5'- or 3'-flanking region of several genes and in the intergenic region of gene clusters, as in the case of the human β -globin gene family²⁸. However, their inclusion in intervening sequences has not been reported. Preliminary data from our laboratory suggest that the human *onc* genes related to feline sarcoma virus (*c-fes*) and avian myelocytomatosis virus (*c-myc*) also contain *Alu* repeated sequences. Further experiments will be required to determine the exact location of these repeats in

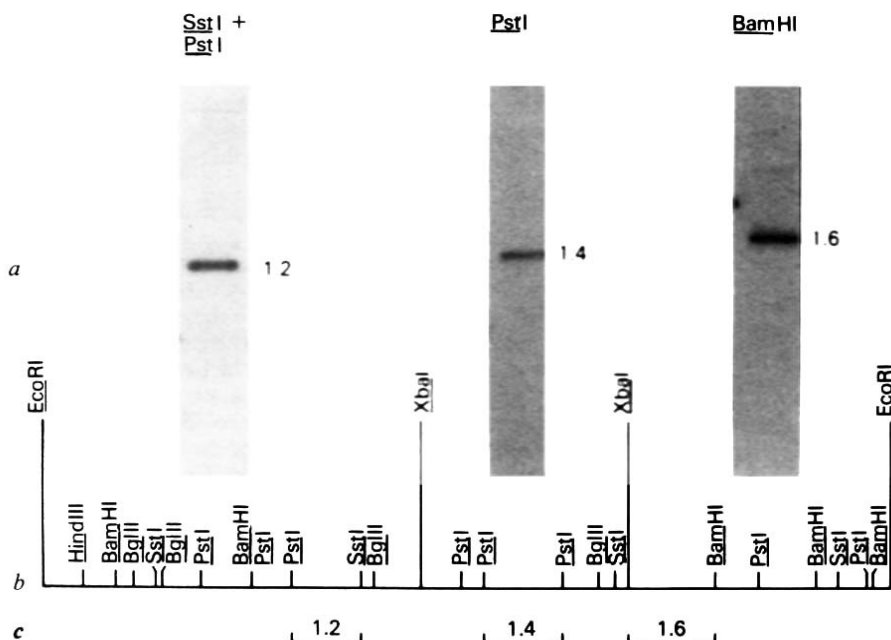


Fig. 6 Mapping in λ -L33 of repeated sequences homologous to the human *Alu* family. *a*, λ -L33 *XbaI* fragments of DNA were prepared as described in Fig. 2 legend and each fragment was hybridized to BLUR 8 DNA²⁵. This recombinant plasmid contains a 300-bp insert corresponding to one copy of the human *Alu* family of interspersed repeats²⁵. *b*, λ -L33 restriction map; *c*, brackets indicate the position of hybridizing fragments (shown in *a*).

the different cellular *onc* genes and their role, if any, in *onc* gene expression.

Thus far, the protein product of either *v-sis* or *c-sis* has not been fully characterized. Many of the known retroviral transforming proteins are kinases which have the unusual property of phosphorylating tyrosine residues²⁹⁻³⁴. Furthermore, recent studies indicate that some of these viral *onc* gene products are structurally and functionally similar to cellular protein kinases involved in the regulation of the (Na⁺ + K⁺)ATPase pump (M. Spector and E. Racker, personal communication). It is of interest to test whether other *onc* genes, including *c-sis*, are involved in this basic metabolic pathway. In preliminary experiments we used cloned SSV DNA bound to nitrocellulose to select *v-sis*-specific mRNA from SSV-transformed non-producer cells. *In vitro* translation of this mRNA resulted in the synthesis of a protein of molecular weight 20,000 (V. Manzari *et al.*, unpublished data). This product could account for the coding potential of *c-sis* and experiments are now being done to characterize the protein.

Substantial evidence suggests involvement of cellular *onc* genes in neoplastic transformation. Increased levels of expression of any of these genes may be the common mechanism in pathogenesis of different types of tumours. This hypothesis is

substantiated by both *in vitro* and *in vivo* experiments. *In vitro* experiments performed with mouse *c-mos*, the cellular homologue of Moloney sarcoma virus²⁷, showed that this gene acquires transforming activity when linked to promoter sequences from the murine leukaemia virus genome²⁷. *In vivo*, B-cell lymphomas in chickens are characterized by abnormal expression of another cellular *onc* gene, *c-myc*¹⁰⁻¹¹. As these genes are conserved structurally and functionally among diverse species it is conceivable that they are also targets for tumorigenesis in man. There is evidence that mutation at certain loci is associated with specific human malignancies³⁵. Moreover, it has been recently reported^{36,37} that DNA from certain human tumours have transformation activity *in vitro* involving specific, possibly unique genetic loci. Complete definition of the family of human *onc* genes will allow us to investigate whether any are involved in the pathogenesis of human tumours. The detection and molecular cloning of the human *c-sis* gene has allowed us to initiate these studies.

We thank Dr T. Maniatis for supplying us with the human DNA library, Dr C. W. Schmid for the BLUR 8 recombinant plasmid and Stefano Martinotti for help with the restriction mapping data.

Received 7 April; accepted 22 April 1981.

- Klein, G. *Viral Oncology* (Raven, New York, 1980).
- Poiesz, B. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 7415-7419 (1980).
- Reitz, M. S., Poiesz, B. J., Ruscetti, F. W. & Gallo, R. C. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Gallo, R. C. 3rd a. *Bristol Myers Symp. Cancer Res.* (eds Kaplan, H. S. & Rosenberg, S. A.) (Academic, New York, in the press).
- Duesberg, P. H. *Cold Spring Harb. Symp. quant. Biol.* **44**, 13 (1980).
- Roussel, M. *et al. Nature* **281**, 452-456 (1979).
- Oppermann, H. D., Levinson, A., Varmus, H. E., Levintow, L. & Bishop, J. M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1804-1808 (1979).
- Karens, R. E., Hayward, W. S. & Hanafusa, H. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3154-3158 (1979).
- Collett, M. S., Erikson, E., Purchio, A. F., Brugge, J. S. & Erikson, R. L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3159-3163 (1979).
- Hayward, W. S., Neel, B. G. & Astrin, S. M. *Nature* **290**, 475-480 (1981).
- Neel, B. G., Hayward, W. S., Robinson, H. L., Fang, J. & Astrin, S. M. *Cell* **23**, 323-334 (1981).
- Spector, D. H., Varmus, H. E. & Bishop, J. M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 410-4106 (1978).
- Goff, S. P., Gilboa, E., Witte, O. N. & Baltimore, D. *Cell* **22**, 777-785 (1980).
- Wong-Staal, F., Dalla Favera, R., Franchini, G., Gelmann, E. P. & Gallo, R. C. *Science* (in the press).
- Theilen, G. H., Gould, D., Fowler, M. & Dungworth, D. L. *J. natn. Cancer Inst.* **47**, 881-889 (1971).
- Wolfe, L. G. *et al. J. natn. Cancer Inst.* **47**, 1115-1120 (1971).
- Gelmann, E. P., Wong-Staal, F., Kramer, R. A. & Gallo, R. C. *Proc. natn. Acad. Sci. U.S.A.* (in the press).

- Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
- Bolivar, F. *et al. Gene* **2**, 95-113 (1977).
- Rigby, P. N., Dieckmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113**, 236-251 (1977).
- Maniatis, T. *et al. Cell* **15**, 687-701 (1978).
- Benton, W. D. & Davis, R. W. *Science* **198**, 180-182 (1977).
- Williams, B. G. & Blattner, F. R. *J. Virol.* **29**, 555-575 (1979).
- Vogelstein, B. & Gillespie, D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 615-619 (1979).
- Jelinek, W. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 1398-1402 (1980).
- Franchini, G., Even, S., Sherr, C. S. & Wong-Staal, F. *Nature* **290**, 154-157 (1981).
- Oskarsson, M., McClements, W. L., Blair, D. G., Maizel, S. V. & Van de Woude, G. F. *Science* **207**, 1222-1224 (1980).
- Duncan, C. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 5095-5099 (1979).
- Witte, O. N., Dasgupta, A. & Baltimore, D. *Nature* **283**, 826-831 (1980).
- Van de Ven, W. J. M., Reynolds, F. H. & Stephenson, J. R. *Virology* **101**, 185-197 (1980).
- Barbacid, M., Beemon, K. & Devare, S. G. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5158-5162 (1980).
- Feldman, R., Hanafusa, T. & Hanafusa, H. *Cell* **22**, 757-765 (1980).
- Collett, M. S. & Erikson, R. L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2021-2024 (1978).
- Sefton, B. M., Hunter, T., Beemon, K. & Eckart, W. *Cell* **20**, 807-817 (1980).
- Knudson, A. G. *New Engl. med. J.* **301**, 606-607 (1979).
- Shilo, B. Z. & Weinberg, R. A. *Nature* **289**, 607-609 (1981).
- Shih, C., Padhy, L. C., Murray, M. & Weinberg, R. A. *Nature* **290**, 261-264 (1981).
- Collins, S. J., Gallo, R. C. & Gallagher, R. E. *Nature* **270**, 347-399 (1977).
- Wong-Staal, F., Reitz, M. S. & Gallo, R. C. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2032-2036 (1979).
- Wahl, G. M., Stern, M. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3683-3687 (1979).
- Ulrich, A. *et al. Science* **196**, 1313 (1977).
- Grunstein, M. & Hogness, D. S. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3961 (1975).
- Davis, R. W., Simon, M. & Davidson, N. *Meth. Enzym.* **24**, 413-428 (1971).

LETTERS TO NATURE

Inflation in the Universe

John D. Barrow*† & Michael S. Turner*‡

* Institute for Theoretical Physics, University of California, Santa Barbara, California 93106, USA

† Department of Physics, University of California, Berkeley, California 94720, USA

‡ Astronomy and Astrophysics Center, University of Chicago, Chicago, Illinois 60637, USA

During the past 15 yr a small but remarkable collection of cosmological conundrums have been identified; each apparently independent, but probably all inter-related at a deeper level than existing theories have penetrated. The outstanding problems are of explaining the observed isotropy, homogeneity, flatness and specific entropy of the Universe¹. Good explanations for the last of these have recently emerged^{2,3} from the study of C, CP and baryon nonconserving interactions which arise in grand unified gauge theories (GUTs) of the strong and electroweak interactions. Recently, Guth has discussed the inflationary Universe as a possible natural explanation for the observed large-scale homogeneity and near critical density ('flatness') of the Universal expansion⁴. We show that one cannot ignore the first conundrum—the isotropy; a Universe

with a large amount of anisotropy will not undergo the inflationary phase. A Universe with only moderate anisotropy will undergo inflation and will be rapidly isotropized. Other consequences of the inflationary Universe are discussed.

The three specific problems which we shall refer to are: the isotropy problem; the homogeneity-horizon problem; and the flatness problem.

The isotropy problem is exhibited by the observed structure of the 2.9 K microwave background radiation⁵. The temperature distribution of this primordial radiation field is isotropic to high precision⁶, $\Delta T/T \leq 10^{-4}$ over large angular scales, $\theta \geq 7^\circ$. All attempts to explain this present isotropy as a consequence of classical dissipative processes acting during the early stages of the Universe have been unsuccessful and they are opposed in principle by various general arguments⁷⁻⁹. Despite being an unstable property of physically realistic cosmological initial conditions, isotropic expansion is a property of the observed Universe. It is not yet clear whether particle production at $t \sim 10^{-43}$ s will help resolve why the present level of isotropy exists and when it arose because some anisotropies grow up again after the Planck time and some anisotropic plane wave universes create no particles¹⁰⁻¹².

The homogeneity-horizon problem falls into two parts. On the one hand, the microwave and X-ray radiation backgrounds,

and the radio source counts^{6,13}, all provide evidence for a striking level of large-scale spatial homogeneity on scales $> \sim 1$ Gpc. But on the other, we see that luminous material is clustered into discrete inhomogeneities of characteristic sizes. Statistical studies⁶ of galaxy clustering indicate the existence of a universal clustering scale, $\sim 10^{15} M_{\odot}$, which divides the developed (non-linear) small-scale structure of galaxies and clusters from the smooth background. On dimensions exceeding the compass of $\sim 10^{15} M_{\odot}$ the observed structure fades away into increasing homogeneity on larger and larger scales. Explaining the origin of small-scale inhomogeneity in the form of galaxies whilst simultaneously explaining the smooth underlying background on very large scales is the homogeneity problem. This problem is compounded by the presence of horizons¹⁴ in known cosmological solutions to Einstein's equations. In the Friedmann model during the radiation-dominated epoch, the total mass of baryons within a causally coherent region of volume $\sim (ct)^3$ at time t after the 'bang' is only $M_H \sim (t/1s)^{1.5} M_{\odot}$. This ensures that causal processes can only smooth out irregularities over the small scale $M_H(t)$ at early times. By the same token, the spontaneous generation of small inhomogeneities during phase transitions or other non-linear events is limited to the scale $M_H(t)$. To explain the large-scale homogeneity or small-scale inhomogeneity, rather than treat them as initial data, it is necessary (but not sufficient) to find mechanisms which can remove or considerably inflate the horizon size during the very early ($t \leq 10^{-4}$ s) stages of the Universe. This is the horizon problem.

It is still an open question whether or not the Universe contains sufficient material to begin recollapsing at a finite time in the future. One reason why this question has proved so difficult to resolve observationally is the 'flatness' problem⁴. The Universe is expanding very close to the intermediate (Einstein-de Sitter) model which contains the critical matter density and so just sufficient expansion energy to expand to infinity. To evolve the observed proximity to the flat (=Euclidean space sections) Einstein-de Sitter model, deviations from the critical density must have been smaller than one part in 10^{57} at the Planck time,

$$\left(\frac{\rho - \rho_{cr}}{\rho_{cr}} \right)_{t_p} \leq 10^{-57}$$

This remarkable degree of fidelity constitutes the flatness problem. Another way of phrasing it is: although the only natural time scale in the gravitation theory is $t_p \sim 10^{-43}$ s, the Universe has expanded for $\sim 10^{60} t_p$ without either recollapsing or entering the Milne (negative) curvature-dominated phase.

Recently, Guth⁴ has suggested a possible resolution of the horizon-homogeneity and flatness problems—the so-called inflationary Universe. In his picture, owing to the slowness of the GUT phase transition at $t_{GUT} \sim 10^{-35}$ s the Universe undergoes a period of de Sitter expansion during which the (particle) horizon grows exponentially with time and the spatial curvature decreases relative to the matter density, also exponentially, thereby explaining two of our conundrums.

In the Friedmann model the expansion scale factor, $R(t)$, is governed by⁵, ($\hbar = c = k_B = 1$);

$$\begin{aligned} \frac{\dot{R}^2}{R^2} &= \frac{8\pi G}{3}(\rho_r + \rho_0) - \frac{k}{R^2} \\ &= \frac{4\pi^3 G}{45} g_*(T) T^4 + \Lambda - \left(\frac{k}{R^2 T^2} \right) T^2 \end{aligned} \quad (1)$$

where ρ_r is the radiation energy density which equals $g_*(T) \pi^2 T^4/30$, $g_*(T)$ is the total effective number of degrees of freedom of all relativistic species at temperature T ($= \sum_{\text{bosons}} g + 7/8 \sum_{\text{fermions}} g$), ρ_0 is the vacuum energy density associated with the cosmological constant, $\Lambda = 8\pi G \rho_0/3$, and $k = \{\pm 1, 0\}$ is the space-time curvature signature⁵. We know today that the cosmological term is small, $\rho_0 < 10^{-30} \text{ g cm}^{-3}$. However, in particle physics theories which undergo spontaneous symmetry breaking (SSB) the Lorentz invariant energy density associated

with the vacuum changes during a phase transition and creates an effective cosmological constant, $\rho_0 \sim O(T_c^4)$, where T_c is the critical temperature of the phase transition. In GUTs the symmetry of the theory breaks down at a temperature $O(10^{15} \text{ GeV})$ leaving an induced cosmological constant and associated vacuum energy density $O(10^{77} \text{ g cm}^{-3})$ —unless one specifies an initial compensatory cosmological term of exactly the same magnitude but opposite sign. In this case, at temperatures above T_c we have $\rho_0 \sim (10^{15} \text{ GeV})^4$, but below T_c the cosmological term is zero—the initial cosmological term and the vacuum energy density associated with SSB precisely cancel.

As the Universe cools below T_c , bubbles of the low-temperature (broken) phase nucleate and grow, and eventually the entire Universe is in the broken phase. However, Guth⁴ pointed out that if the nucleation rate is sufficiently small, then the Universe will remain in the high-temperature (symmetric) phase for a non-negligible period: the 'false vacuum' is metastable. During this interval the initial cosmological term, ρ_0 , remains uncanceled and soon begins to dominate the expansion dynamics, $\rho_0 > \rho_r$. The Universe undergoes a phase of de Sitter expansion, $R \propto \exp(\Lambda^{1/2} t)$ with $T \propto R^{-1} \propto \exp(-\Lambda^{1/2} t)$ and 'supercools' (the Universe remains in the symmetric vacuum until a temperature $T_s \ll T_c$). Finally, the transition is completed ($t = t_* \geq t_{GUT}$ and $T_s \sim T_c \exp(-\Lambda^{1/2} t_*) \ll T_c$); the initial ρ_0 term is cancelled and the Universe is reheated to nearly T_c by the latent heat release. The expansion then resumes Friedmann behaviour. (The graceful return to a Friedmann model remains a difficulty⁴.)

Just before the transition occurs, ($T \geq T_c$), one might expect that because t is not too different from $t_p \sim 10^{-43}$ s, (the only natural time scale in the theory), the curvature term (k/R^2) would be $\sim O(\rho_r)$ (the flatness problem). In addition, if the baryons were created (or already present) at this time, then there would be only about one baryon per horizon volume. Guth⁴ points out that if the inflationary phase lasts long enough to supercool by a factor $\sim 10^{28}$ ($t_* \sim 65 \Lambda^{-1/2} \sim 65 t_{GUT}$), then when Friedmann behaviour recommences: (1) the curvature term will have been reduced relative to the radiation density by a factor $\sim 10^{56}$ ($T_* \sim T_c$, while R has inflated by 10^{28}) so solving the flatness problem; and (2) the horizon has grown large enough to encompass the portion of the Universe accessible to us today $\geq 10^{22} M_{\odot}$. Thus if the Universe was smooth on scales of the horizon at t_{GUT} , the horizon problem is solved.

The question naturally arises as to whether nucleation is slow enough to allow such supercooling. The probability that a given point remains in the unbroken phase is (ref. 4, equations 3.14/.15)

$$p(t) = \exp \left\{ - \int_0^t dt_1 \lambda(t_1) R^3(t_1) V(t, t_1) \right\} \quad (2)$$

where

$$V(t, t_1) = \frac{4\pi}{3} \left(\int_{t_1}^t \frac{d\theta}{R(\theta)} \right)^3$$

and λ is the nucleation rate which, if one assumes that $\lambda(t) = \lambda_0$ and $R \propto \exp(\Lambda^{1/2} t)$, then implies $p(t) = \exp(-t/\tau)$ where $\tau = 3\Lambda^{3/2}/4\pi\lambda_0$. In principle λ_0 can be computed and Guth states⁴ that because $\lambda_0 \approx A\rho_0 \exp(-B)$, one can easily obtain $\tau \approx t_* > 65 \Lambda^{-1/2}$; (note, $A \sim O(1)$ and B is a barrier penetration term).

This analysis relies on the assumption of Friedmann expansion for $R(t)$; that is, it resolves the homogeneity-horizon and flatness problems by ignoring the isotropy problem. The Friedmann model is already isotropic and homogeneous. It is argued⁴ that particle physics processes homogenize the Universe on scales $\leq ct_{GUT}$ so the Universe can be assumed 'patchwise' homogeneous. However, the tacit assumption of some degree of isotropy remains. We shall show that if it is relaxed the inflationary phase can be prevented.

If the very early Universe were anisotropic then a good description of its expansion dynamics is provided by a generalized Friedmann equation¹⁵ for the (geometric) mean scale factor

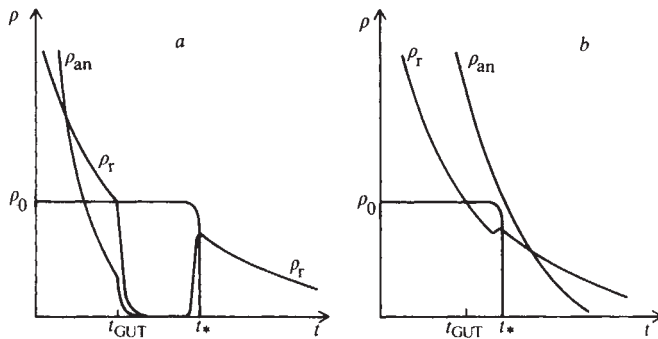


Fig. 1 The contributions to the right-hand side of equation (3) are shown as a function of time. *a*, If $\rho_{an} \leq \rho_0$ at t_{GUT} , the inflationary phase will ensue and both ρ_r and ρ_{an} decrease exponentially. When the phase transition is complete, $t = t_*$, the latent heat release increases ρ_r to $\sim T_c^4$. *b*, If $\rho_{an} \geq \eta^{1/2} \rho_0$ at t_{GUT} , the inflationary phase does not occur, and the Universe continues to be anisotropic. When the transition is complete, there is a latent heat release increasing ρ_r slightly. The anisotropy is always decaying adiabatically ($\rho_r/\rho_{an} \sim R^2$), eventually becoming negligible.

$R(t)$,

$$\frac{\dot{R}^2}{R^2} = \frac{8\pi G}{3}(\rho_r + \rho_0) - \frac{k}{R^2} + \frac{\Sigma^2}{R^6} \quad (3)$$

where $\rho_{an} = \Sigma^2 R^{-6}$ is the anisotropy (gravitational wave) 'energy density'. Equation (3) is exact for space-times which possess isotropic three-curvature and an excellent approximation to those that do not over short-time intervals. In equation (3) the constant Σ is arbitrarily fixed by the initial data. When $8\pi G\rho/3 < \Sigma^2 R^{-6}$ the cosmological model is called anisotropic and will have an overall expansion rate, $R \propto t^{1/3}$, which differs from the isotropic radiation model, $R \propto t^{1/2}$. We can choose a class of anisotropic models which have Σ large enough to remain anisotropic until any pre-specified epoch. The isotropy problem can be thought of as explaining why Σ must be chosen to be 'small'. All anisotropic models described by equation (3) eventually isotropize as $\rho_r/\rho_{an} \propto R^2$. They will only have isotropized by t_{GUT} if Σ is specially chosen.

If the Universe expands anisotropically at t_{GUT} then we can solve equation (3) to obtain a description of the competition between anisotropy and vacuum energy (Λ)

$$R^3 = \frac{\Sigma}{\Lambda^{1/2}} \sinh(3\Lambda^{1/2}t) \quad (4)$$

We obtain the Kasner anisotropic behaviour, $R \sim t^{1/3}$, when the anisotropy dominates ($t \rightarrow 0$) and de Sitter, $R \sim \exp(\Lambda^{1/2}t)$, when the vacuum energy dominates ($t \rightarrow \infty$).

If the anisotropy energy density ρ_{an} is less than ρ_0 ($\sim \rho_r$) at t_{GUT} , then the inflationary phase will proceed (Fig. 1*a*). In addition ρ_{an} will decay adiabatically as $\exp(-6\Lambda^{1/2}t)$, rapidly degrading any anisotropies. However, if $\rho_{an} > \rho_0$ at t_{GUT} , then the Universe will continue to be anisotropic with $R \propto t^{1/3}$ and $\rho_{an} \propto t^{-2}$. If the transition is slow enough, then ρ_{an} may fall below ρ_0 and an inflationary phase would ensue. To address this issue consider $p(t)$ given by equation (1). For $R \sim t^{1/3}$ we find

$$p(t) = \exp(-t/\tau) \quad (5)$$

$$\tau = \frac{80}{9\pi\lambda_0 t^3} \quad (6)$$

In computing τ we have assumed spherical geometry, although the actual geometry is nonspherical. With Kasner geometry τ is smaller by a geometric factor of 0(10). Therefore, we shall use $\tau = 1/\pi\lambda_0 t^3$ rather than equation (6). Unlike the inflationary case, where $\tau = 3\Lambda^{3/2}/4\pi\lambda_0 \approx 3/4\pi\lambda_0 t_{GUT}^3$ the nucleation time scale τ decreases as $\sim t^{-3}$ so that $t_* \sim (\pi\lambda_0)^{-1/4}$. Suppose that in the absence of anisotropy $t_* = \eta t_{GUT}$, then in the presence of anisotropy $t_* \approx \eta^{1/4} t_{GUT}$. During the time between t_{GUT} and t_* , ρ_{an} will have decreased by a factor $(t_*/t_{GUT})^2 \approx \eta^{1/2}$. For $\eta \sim 65$,

(the value required to resolve the horizon problem in the isotropic model⁴), ρ_{an} has only decreased by a factor ~ 10 by t_* . That is, if $\rho_{an} \geq 10\rho_0$ at t_{GUT} , then the anisotropy will prevent the occurrence of the inflationary phase (Fig. 1*b*). The value of the nucleation rate λ_0 is very uncertain, so that η might be $\gg 65$ or $\ll 65$; in any case, one must not separate the question of isotropy from the homogeneity-horizon and flatness problems.

The inflationary Universe was motivated, at least in part, by the flatness and homogeneity-horizon conundrums. However, the isotropy problem must also be considered. The presence of a large amount of initial anisotropy $\rho_{an}(t_{GUT}) \geq \eta^{1/2} \rho_0(t_{GUT})$, will prevent the de Sitter phase from occurring and will decay away adiabatically thereafter ($\propto R^{-6}$). The pressure of this anisotropy at t_{GUT} will not prevent baryosynthesis, and in fact allows for the production of isothermal perturbations¹⁶ which are highly desirable for galaxy formation^{6,17}; nor would this anisotropy interfere with nucleosynthesis or adversely affect the isotropy of the 2.9 K background.

If the inflationary phase were to occur there would be some interesting consequences. Any relics from an earlier epoch are effectively erased. For example, any initial baryon or lepton number is deleted (hence baryosynthesis must occur after the inflation which is not a difficulty as the Universe is reheated to $\sim T_c$); any gravitons emerging from t_p will have their density degraded relative to photons by a massive factor $\exp(3\Lambda^{1/2}t_*) \sim 10^{83}$ because the latent heat release will not couple to the collisionless graviton sea. Recently, Starobinski^{18,19} has pointed out that the graviton background arising from a cosmological model with a de Sitter expansion before t_p (not arising from a phase transition) is potentially detectable at a frequency of $\sim 10^{-4}$ Hz. If this radiation were detected it would exclude the inflationary process by showing that the primordial graviton density is not $\sim 10^{80}$ times smaller than the photon density.

It also seems that, whilst solving the horizon problem, the inflationary Universe may compound the part of the homogeneity problem which seeks an explanation for the existence of small inhomogeneities and thereby galaxies. Any curvature fluctuations (spatial variations in k/R^2) imprinted on the dynamics before t_{GUT} would have their effective amplitudes reduced by $\sim \exp(2\Lambda^{1/2}t_*) \sim 10^{56}$. A new spectrum of adiabatic inhomogeneities would have to be produced when the phase transition is completed (note, anisotropies in the spatial three curvature are also reduced during the de Sitter phase). In essence the inflationary Universe cleans the slate, and the Universe is reborn. Elsewhere¹⁶ we have described a way of generating isothermal fluctuations during this epoch. The process of bubble nucleation and percolation is inhomogeneous and might provide a mechanism for creating new fluctuations; for example, bubble wall collisions might produce large-scale inhomogeneous shear, which in turn can give rise to isothermal perturbations¹⁶. However, owing to its extremely chaotic nature these fluctuations might be of such large amplitude on all scales up to the inflated horizon size $\geq 10^{22} M_\odot$, that a profusion of black holes would result, rather than a regular Poisson spectrum.

In the Guth hypothesis⁴ the explanation of the flatness, and homogeneity, problems is tied to that of the isotropy problem. The web of interconnections may be far more extensive: a theory of quantum gravity may reveal them all to have a common origin^{10,11}. An interesting inter-relationship is already known at the classical level. It has been shown^{8,20} that the set of homogeneous initial data giving rise to open isotropic universes asymptotically is of measure zero in initial data space but the set which gives rise to flat, isotropic Universes is not of measure zero. When the initial anisotropy of the Universe is not too large, the inflationary Universe can exist and explain the flatness we observe. This case is an interesting contrast to the result of Collins and Hawking: inflated universes will be flat if they are isotropic.

We thank all the members of the Early Universe Program at the Institute for Theoretical Physics, UCSB, for helpful discussions. M.S.T. thanks Ira Wasserman and Henry Tye for helpful discussions. This work was supported in part by the

Institute for Theoretical Physics (NSF grant PHY77-27084), the Miller Institute for Basic Research and DOE grant AC02-80ER10773.

Received 5 March; accepted 28 April 1981.

1. Barrow, J. D. *Sci. Prog.* **65**, 129–60 (1978); *Problems of the Cosmos* (Encic. Italiana, Rome, 1980).
2. Kolb, E. & Wolfram, S. *Phys. Lett.* **91B**, 217 (1980); *Nucl. Phys.* **B172**, 224 (1980).
3. Fry, J. N., Olive, K. A. & Turner, M. S. *Phys. Rev. Lett.* **45**, 2074 (1980); *Phys. Rev.* **D22**, 2953; **D 22**, 2977 (1980).
4. Guth, A. *Phys. Rev.* **D23**, 347 (1981).
5. Weinberg, S. *Gravitation and Cosmology* (Wiley, New York, 1972).
6. Peebles, P. J. E. *The Large Scale Structure of the Universe* (Princeton University Press, 1980).
7. Barrow, J. D. & Matzner, R. A. *Mon. Not. R. astr. Soc.* **181**, 719–28 (1977).
8. Collins, C. B. & Hawking, S. W. *Astrophys. J.* **180**, 317 (1973).
9. Stewart, J. M. *Mon. Not. R. astr. Soc.* **145**, 347 (1969).
10. Lukash, V. N., Novikov, I. D., Starobinskii, A. A. & Zeldovich, Ya. B. *Nuovo Cim.* **35B**, 293–397 (1977).
11. Hu, B. L. & Parker, L. *Phys. Rev.* **D17**, 933 (1978).
12. Gibbons, G. W. *Commun. Math. Phys.* **45**, 191–202 (1975).
13. Webster, A. *Mon. Not. R. astr. Soc.* **175**, 61; 71 (1976).
14. Rindler, W. *Mon. Not. R. astr. Soc.* **116**, 663 (1956).
15. Ellis, G. F. R. *Cargèse Lectures in Physics* Vol. 6, (ed. Schatzman, E.) (Gordon & Breach, New York, 1973).
16. Barrow, J. D. & Turner, M. S. Preprint (80/03 ITP, 1981); *Nature* **291**, 469 (1981).
17. Barrow, J. D. *Phil. Trans. R. Soc.* **A296**, 273 (1980).
18. Starobinskii, A. A. *Soviet JETP Lett.* **30**, 719 (1979).
19. Lukash, V. N. *Soviet JETP Lett.* **31**, 596 (1980).
20. Barrow, J. D. & Tipler, F. J. *Nature* **276**, 453 (1978).

Evidence for sulphur implantation in Europa's UV absorption band

Arthur L. Lane, Robert M. Nelson
& Dennis L. Matson

Jet Propulsion Laboratory, California Institute of Technology,
Pasadena, California 91109, USA

The International Ultraviolet Explorer (IUE) spacecraft has obtained observations of the galilean satellites over the past 2 years which fortuitously span the periods of the Voyager encounters with the jovian system. Our IUE observing programme is designed to determine the UV spectral characteristics of the galilean satellites as a function of orbital position, large-scale areal variability and temporal dynamics. During the past year we have concentrated on the albedo variations of each body^{1,2}. We report here the discovery of an absorption feature at 280 nm in Europa's reflection spectrum. Observations with the IUE show that this absorption is strongest on Europa's trailing hemisphere (central longitude 270°). We identify the feature as an SO₂ absorption band and hypothesize that SO₂ may form when energetic jovian magnetospheric sulphur ions are injected into Europa's water-ice surface.

Initial analysis of the Europa spectra² showed a substantial near-UV (250–320 nm) albedo difference when comparing the leading (central longitude 90°) and trailing (central longitude 270°) hemispheres. This effect has been reported previously on the basis of ground-based observations^{3–5}. In view of these earlier discoveries we did not consider the near-UV behaviour as anomalous. A more detailed examination of the low-resolution (~1.1 nm FWHM) IUE spectra showed a pronounced decrease of disk-averaged albedo towards shorter wavelengths (3,250–2,600 Å). The decrease was observed for both the leading and trailing hemispheres. To improve the signal-to-noise ratio, several spectra from the same hemispheres (orbital longitudes) were summed. These pairs of summed spectra represent the extrema of the visible and UV albedo variations. These sets were then ratioed to each other. For Europa, spectra at orbital longitudes between 84° and 104° were used for the leading side and between 270° and 281° for the trailing side. For comparison, a set centred near 90° and 270° was selected for Ganymede (see Fig. 1). Noise which results from partial pixel misregistration and from small wavelength drifts in the extracted data from the IUE image tube has not been filtered or smoothed. Therefore,

we present a 'worst-possible' case for the credibility of the ratioed spectra. Unlike the trailing/leading side comparison for Ganymede which demonstrates an additional darkening with decreasing wavelength on the trailing side relative to the leading side, the Europa ratio has a definite absorption band centred near 280–290 nm. Modest amplification of the Ganymede ratio to the noise limit of the data shows no similar band.

The shape of the Europa absorption band appeared suspiciously similar to the absorption spectrum of the $\tilde{A}-\tilde{X}$ UV band of SO₂ centred between 280 and 290 nm (ref. 6). This portion of the SO₂ absorption spectrum is also presented in Fig. 1. The alignment and shape of the vapour-phase SO₂ absorption spectrum is an excellent match to the observed absorption on the trailing side of Europa. We suggest that on Europa this spectral feature is caused by a sulphur atom–oxygen atom interaction within the water-ice lattice. It is not caused by an SO₂ surface frost, because the frost has a very different UV spectrum that does not match what has been observed⁷. Also, unlike Io, where a 4.08- μ m absorption band assigned to SO₂ frost has been observed, no SO₂ IR band has been detected on Europa^{8,9}. There is no evidence for a low-pressure SO₂ atmosphere, from either Voyager observations (A. L. Broadfoot, personal communication) or light curves of mutual satellite eclipses and occultations. If the imbedded sulphur atoms are sufficiently distant from each other in the ice lattice, they may acquire the bulk spectral character of a 'gas' in which the molecular interactions are small. This is the case for the present model. For the concentrations of sulphur detected, each deep-imbedded sulphur atom has between 120 and 200 Å³ of volume, at least an order of magnitude greater than the molecular volume found in a frost.

The possibility for the presence of the element sulphur is supported by other evidence. Data from the Voyager plasma experiment^{10,11}, and the low-energy charged particle (LECP) experiment¹², show that the galilean satellites are in an ion plasma composed primarily of sulphur and oxygen in which the co-rotating ions reach keV energies. The Voyager plasma experiment (J. Sullivan, personal communication) provided a measurement of about 7 sulphur ion cm⁻³ for the 'lower-energy' ions at Europa's orbit distance of 9.4 R_J. In this region their mean velocity is ~100–115 km s⁻¹. If we remove Europa's orbital velocity of ~13.7 km s⁻¹, then the mean flux of sulphur ions onto Europa's trailing side is ~7 × 10⁷ ion cm⁻² s⁻¹. Data from the LECP experiment indicate that there are also higher-energy sulphur and oxygen ions present, with energies in the hundreds of keV (ref. 12). An estimate of the integrated flux of these S and O ions is ~10⁸ cm⁻² s⁻¹ with a mean energy of 100 keV (ref. 13, R. Johnson, personal communication). Thus, we can model the sulphur ion flux at Europa with two distinct distributions: a 1-keV source with a flux of ~7 × 10⁷ ion cm⁻² s⁻¹ and a 100-keV source with a flux of ~3 × 10⁷ ion cm⁻² s⁻¹. Although laboratory data for heavy ion bombardment of water ice are meagre, R. Johnson and P. Haff (personal communications) have independently estimated that the low, 1-keV sulphur particles should penetrate the ice surface to a depth of ~35–40 Å (about 10 molecular layers of water ice in the I hexagonal form). The 100-keV ion penetration is more difficult to estimate, but a linear model for depth versus energy seems reasonable until laboratory data are available. The faster ions may penetrate to between 3,000 and 4,000 Å.

In addition to deposition, the heavy ion impacts produce sputtering of the surface ice and over long periods of time there is erosion and the possibility for removal of a significant mass of water ice from Europa. Sputtering in water ice is anomalous when compared with processes for metals; there is an unexpectedly large yield of sputtered fragments from ice^{14,15}. The referenced studies showed that the sputtering yield is dependent on the mass of the ion as well as its energy. Johnson *et al.*¹³ and Eviatar *et al.*¹⁶ have modelled the erosion at Europa caused by sulphur and oxygen ions diffusing outwards from Io's plasma torus. The erosion rates estimated are as high as 100 m of ice per 10⁹ yr, which corresponds to 10³ Å yr⁻¹.

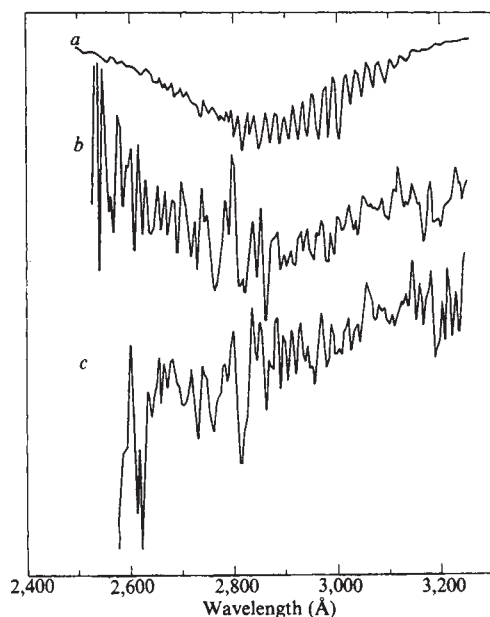


Fig. 1 *a*, The gas-phase absorption spectrum of the $\tilde{A}$ - $\tilde{X}$ band of SO_2 (ref. 6). The 10% attenuation illustrated in the depth of the band corresponds to a column density of 1×10^{17} molecules cm^{-2} . *b*, Three separate Europa trailing side and leading side spectra were added before the ratio spectrum was formed. Each separate trailing side spectrum shows $\sim 3\%$ absorption. Note that the brightness ratio of the two hemispheres is approximately constant at 2,600 and 3,200 Å. Thus, ratio spectrum is not seriously distorted. *c*, This trailing side/leading side ratio spectrum of Ganymede was formed the same way as the Europa ratio spectrum. The additional darkening of Ganymede's trailing side is seen in the gradual decrease of the ratio. No absorption minimum is detectable within the noise limit of the data. We conclude that this indicates on Europa the presence of S-O bands in a water-ice matrix, originating from sulphur ion implantation.

The ability to see sulphur deposition depends on the balance between injection/deposition and erosional loss to space or ballistic migration to the opposite side of Europa. The leading side of Europa has no detectable absorption band; the absorption would be marginally detectable, in our present limits, at a factor of about 4 less than what is present on the trailing side. Loss to space may be significant, but no atmosphere or strong outgassing signature was detected by either Voyager 1 or 2. The lack of a positive atmospheric detection by the Voyager UV spectrometer makes high erosion rates unlikely. Also, high erosion rates would reach the 3,000–4,000 Å deep sulphur ions within days to months of their initial injection into the ice surface. Within that time $\sim 10^{12}$ – 10^{14} new 'deep-seated' ions per cm^{-2} would have been injected into the ice, and all the low-energy ions lying in the upper 50 Å would have been removed. Even 10^{14} sulphur atoms (as SO_2) would not be detectable.

The depth of the Europa absorption band can be estimated from the individual spectra that make up the trailing side data set: $\sim 3\%$ attenuation within a factor of 2. To produce such an absorption, $\sim 2 \times 10^{16}$ molecules of SO_2 per cm^2 are required. Erosion rates of 10^4 – 10^6 Å yr^{-1} are about 2–4 orders of magnitude too large to retain sufficient sulphur atoms at a depth where an S-O molecular spectral band can be established and maintained. In fact, not all sulphur ions will make spectroscopically active bonds, so the inequality could be even larger. The fate of the lower-energy sulphur ions nearer the surface is unknown. They may be lost to space through energetic sputtering, or more probably, they co-deposit with any water molecules and oxygen atoms somewhere else on Europa after following ballistic trajectories from the sputtering site. Whether these sulphur atoms end up as sulphur dioxide molecules buried in a deposited

oxygenated water ice is also unknown. No laboratory data seem to be available for 2,800 Å light penetration into highly irregular water ice in which the many optical scattering centres far exceed the number of SO_2 molecules. If this ice deposit is relatively opaque, any real SO_2 could be hidden from detection. High erosion rates seem to be a problem for the detection of this absorption band. L. J. Lanzerotti has indicated that laboratory experimental studies seem to indicate that energetic particle bombardment of amorphous ice causes a conversion to a crystalline structure. We have assumed that the UV penetration into the surface ice is one or two wavelengths deep (3,000–5,000 Å) because of the optical scattering centres and lattice defects. For an amorphous ice, this assumption should be adequate. Regular crystalline ice may permit UV penetration to 10,000 Å or more based on the fact that liquid water is quite transparent to 2,800 Å light. If the surface were crystalline ice, we would be seeing absorptions caused by the deepest implantations, and would bias our sulphur-ion source to a higher-energy distribution.

The lower rates of Johnson *et al.*¹³ and the revised rates of Eviatar *et al.*¹⁶ seem more promising. At 10^3 Å yr^{-1} , it would take 3–4 yr to reach the deeper-lying sulphur atoms in the damaged water-ice lattice. The equilibrium implanted sulphur concentration would be of the order of $3\text{--}4 \times 10^{15}$ cm^{-2} , or only a factor of 5 less than what was detected. Johnson *et al.*¹³ state that the rate could be as high as 100 m per 10^9 yr. If it were closer to 20 m, then an approximate equilibrium between deposition and erosion is possible. Even in this case, the fate of the low-energy, 1-keV sulphur ions that are stopped in the upper 40 Å is unclear. About 5×10^{14} sulphur ions cm^{-2} should be an equilibrium value with an erosion rate of 20 m per 10^9 yr (0.2 yr to deplete), and this number is about 10 times smaller than the current limit of IUE detectivity. These sulphur atoms could be buried in water-ice fragments that strongly scatter UV light and thus, not be detectable by UV absorption techniques.

We conclude that the IUE has detected an absorption band on the trailing side of Europa that resembles SO_2 , and seems to result from S-O bond formation between deeply implanted sulphur atoms and the adjacent, damaged water-ice lattice. The sulphur detected seems to come from the energetic (hundreds of keV) sulphur ions that are present in the jovian magnetosphere. Given the current state of knowledge about the physical processes involved in heavy ion bombardment in water ice, we cannot be precise about the fate of much of the sulphur which undergoes sputtering along with the dominant water-ice fragments. An appropriate equilibrium condition can be found to match the observed spectral data if sputtering erosion occurs at no greater than 20 or so metres per 10^9 yr.

We thank the IUE observatory staff for support during the acquisition of these data. A.L.L. thanks R. Johnson, P. Haff, A. Eviatar and T. Johnson for helpful discussions, and L. J. Lanzerotti for a helpful review. This work represents one phase of research performed at the Jet Propulsion Laboratory, California Institute of Technology under NASA contract 7-100.

Received 3 November 1980; accepted 10 April 1981.

- Nelson, R. M. *et al. Science* **210**, 784 (1980).
- Nelson, R. M., Matson, D. L., Lane, A. L., Motteler, F. C. & Ockert, M. E. *Bull. Am. astr. Soc.* **12**, 713 (1980).
- Nelson, R. M. & Hapke, B. W. *Icarus* **36**, 304 (1978).
- Morrison, D. & Morrison, N. D. *Planetary Satellites* (ed. Burne, J.) 363 (University of Arizona Press, 1977).
- Johnson, T. V. *Icarus* **14**, 94 (1971).
- Herzberg, G. *Molecular Spectra and Molecular Structure* Vol. 3, 605 (Reinhold, New York, 1966).
- Nash, D. B., Fanale, F. P. & Nelson, R. M. *Geophys. Res. Lett.* **11**, 665 (1980).
- Nash, D. B. & Nelson, R. M. *Nature* **280**, 763 (1979).
- Fanale, F. P. *et al. Nature* **280**, 761 (1979).
- Bridge, H. S. *et al. Science* **204**, 987 (1979); **206**, 972 (1979).
- Bridge, H. S., Sullivan, J. D. & Bagenal, F. *Nature* **280**, 798 (1979).
- Krimigis, S. M. *et al. Science* **204**, 998 (1979); **206**, 977 (1979).
- Johnson, R. E., Lanzerotti, L. J., Brown, W. L. & Armstrong, T. P. *Science* (submitted).
- Brown, W. L., Lanzerotti, L. J., Poate, J. M. & Augustyniak, W. M. *Phys. Rev. Lett.* **40**, 1027 (1978).
- Brown, W. L. *et al. Nucl. Instrum. Meth.* **170**, 321 (1980).
- Eviatar, A., Siscoe, G. L., Johnson, T. V. & Matson, D. L. *Icarus* (submitted).

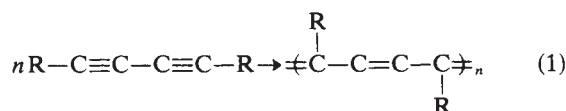
Vapour deposition polymerization of butadiyne

Arthur W. Snow

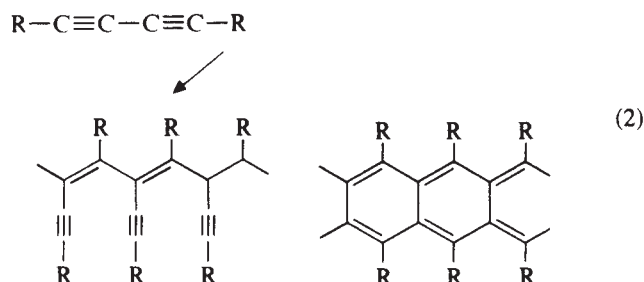
Chemistry Division, Naval Research Laboratory,
Washington DC 20375, USA

In seeking preparative routes to highly conjugated polymeric systems, we have found that butadiyne, C_4H_2 , the simplest diacetylene, may be polymerized from the vapour state by selective deposition onto an organic substrate polymer surface, such as polyethylene or Teflon. This polymerization is sensitive to the nature of the substrate surface, occurring most rapidly on a hydrocarbon surface. The polymer is characterized by IR spectroscopy as having a polyconjugated main chain with pendant acetylenic functional groups. ESR spectroscopy shows the polymer to be highly paramagnetic which is interpreted as indicating the presence of a large number of defects within the conjugated polymer. Structurally, this butadiyne polymer appears to be more closely related to substituted diacetylene polymers prepared by melt polymerization as opposed to those prepared by solid state polymerization.

In the solid state, mechanistic investigations with single crystal diacetylene polymerizations have elucidated monomer molecular and crystal structure requirements as well as a 1,4 polymer repeat unit structure¹⁻³:



In the melt^{4,5} or in solution^{5,6}, the polymerization is more complex, and polymer structures are less extensively characterized with combination of structures having been proposed^{4,5}:



The presence of the pendant group obscures much characterization information with regard to the polymer chain structure. The butadiyne ($R=H$) polymer, in addition to being conveniently prepared and handled as a vapour-deposited film, presents a unique opportunity for characterizing the chain structure.

Depending on the nature and composition of the substrate surface, these polymerizations may be conducted by thermal

initiation at temperatures ranging from 25 to 100 °C. Polymerization occurs most rapidly on a hydrocarbon surface but fluorocarbon surfaces can also be coated at reasonable rates. For example, in an atmosphere of 700 mm C_4H_2 at 25 °C, polyethylene film 3.7 thousandths of an inch thick, will increase its weight by 20% from polymer deposited over a 6-h period while a Teflon film 2.1 thousandths thick, subjected to a similar exposure for 36 h, increased its weight by 10%. Other surfaces such as quartz, glass, sodium chloride or aluminium oxide are highly inert showing only trace or no polymerization at all after a month's exposure to the same conditions. However, at 100 °C it is possible to deposit films on their surfaces. It is interesting that a simple hydrocarbon monomer should selectively polymerize on hydrocarbon or fluorocarbon surfaces. The polymer conforms well to the substrate surface, and, in the case of organic substrates, examination of a cut cross-section of the sample, shows some penetration of the substrate film by the polybutadiyne does occur. It is also noteworthy that the deposited polymer like the C_4H_2 monomer is a hydrocarbon, and, as might be anticipated, an accelerating polymerization rate is observed with a substrate such as Teflon. As might also be expected, the monomer will polymerize rapidly in the bulk liquid state. At high degrees of conversion this bulk liquid polymer has a brass-like metallic lustre similar to some substituted diacetylene single crystal polymers.

Characterization is made difficult by total insolubility in all organic solvents as well as concentrated sulphuric acid and 10% hydroxide. Consequently, it is necessary to use solid-state techniques.

In this polymerization system the growth of a very intense singlet ESR signal accompanies the polymerization of the liquid or gaseous monomer. The signal has a free electron g -value, a linewidth of 10 G and a lorentzian line shape. Similar ESR spectra have been reported for solid state⁸⁻¹⁰, melt^{4,5} and solution^{6,7} polymerized substituted diacetylenes. However, the spin density of the melt polymerized diacetylene is significantly greater than that observed for solid state (10^{19} spin/g compared with 10^{16} spin/g). A free electron ESR singlet is frequently observed in polyene systems and is usually ascribed to a defect in the conjugated polymer chain¹¹. The higher spin density may be correlated with a larger number of defects. Vapour deposited polybutadiyne has a spin density of 8×10^{19} spin/g.

IR spectroscopy yields the most structural information. The collection of bands at 3,315, 2,090 and 640 cm^{-1} (Fig. 1) indicate the presence of a high concentration of pendant terminal acetylenic functional groups. The broad bands at 1,600, 1,210 and 885 cm^{-1} suggest that the polymer chain is unsaturated and polydisperse. In the context of this polymerization, the band at 885 cm^{-1} could be correlated with a central acene structure or, alternately, with the C—H vibrations of a 1,3,5-trisubstituted benzene structure¹². In describing the melt polymerized diphenyldiacetylene and dimethyldiacetylene systems, the former correlation in addition to other data has been used to propose a block polyene-polyacene copolymer structure composed of the products in equation (2). In the present case of unsubstituted butadiyne with direct detection of a high density of terminal acetylenic functional groups, a substituted polyphenyl is being considered as well.

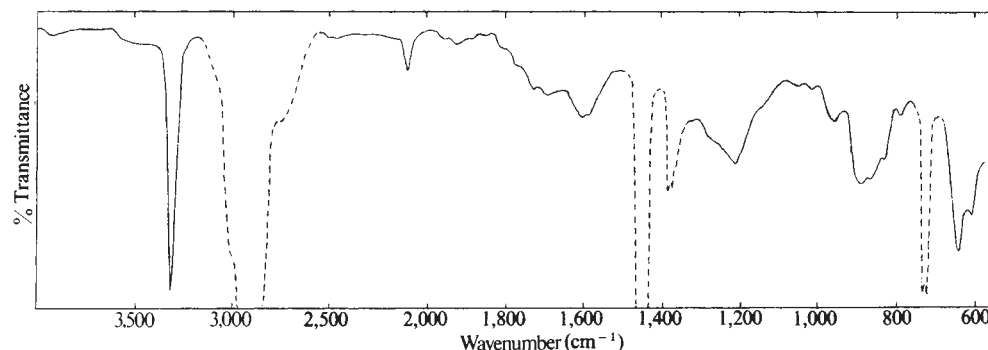
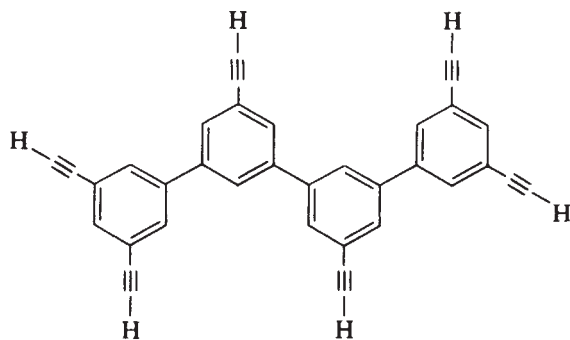


Fig. 1 Transmission IR spectrum of polybutadiyne deposited on a polyethylene film. The broken line corresponds to absorptions from the polyethylene.



The observation that the polybutadiyne penetrates into the substrate film suggests that absorbed monomer may diffuse through the surface before polymerizing. Polymer formation might be envisioned as involving formation of a substituted linear polyene structure followed by a partial intramolecular cyclization yielding structures represented in equation (2) or involving a trimerization of terminal acetylenic groups of two monomers and of a growing polymer molecule to produce a polymer with phenyl linkages in the main chain and pendent unreacted terminal acetylenic groups. The intense ESR signal suggests many defects exist in such a conjugated system. The kinetics, structure and properties of this deposition polymeric system are currently under study and will be reported elsewhere.

I thank the City University of New York, College of Staten Island for use of their ESR facility, and Professor N.-L. Yang for helpful discussions.

Received 10 February; accepted 11 May 1981.

1. Wegner, G. *Makromolec. Chem.* **154**, 35–48 (1972); **145**, 85–94 (1971); *J. polym. Sci. B* **9**, 133–144 (1971).
2. Baughman, R. H. *J. polym. Sci. A212*, 1511–1535 (1974).
3. Bloor, D., Koski, L., Stevens, G. C., Preston, F. H. & Ando, D. *J. mater. Sci.* **10**, 1678–1688 (1975).
4. Berlin, A. A., Cherkashin, M. I., Chauser, M. G. & Shifrina, R. R. *Vysokomolekul. Soedin* **A9**, 2219–2225 (1967).
5. Chauser, M. G., Cherkashin, M. I., Kurhnerev, M. Ya., Protsuk, T. I. & Berlin, A. A. *Vysokomolekul. Soedin* **A10**, 916–924 (1968).
6. Wiley, R. H. & Lee, J. Y. *J. macromolec. Sci. A5*, 513–527 (1971).
7. Teyssié, P. H. & Korn-Girard, A. C. *J. polym. Sci.* **2**, 2849–2858 (1964).
8. Baughman, R. H., Exarhos, G. J. & Risen, W. M. *J. polym. Sci. A212*, 2189–2193 (1974).
9. Stevens, G. C. & Bloor, D. *J. polym. Sci. A213*, 2411–2427 (1975); *Chem. phys. Lett.* **40**, 37–40 (1976).
10. Eichele, H., Schwoerer, M., Huber, R. & Bloor, D. *Chem. phys. Lett.* **42**, 342–346 (1976).
11. Bishop, A. R. *Solid State Commun.* **33**, 955–960 (1979).
12. Avram, M. & Mateescu, G. H. *Infrared Spectroscopy*, 211; 218–220 (Wiley-Interscience, New York, 1972).

Negative exotic particles as low-temperature fusion catalysts and geochemical distribution

Christian K. Jørgensen

Département de Chimie Minérale, Analytique et Appliquée,
University of Geneva, CH 1211 Geneva 4, Switzerland

Minute concentrations of unfamiliar particles (X^+ or X^-) with atomic weights M between 10^2 and 10^6 (10^{11} and 10^{15} eV) may be remnants^{1–4} of the Big Bang or may originate in recent events (their expected properties are discussed elsewhere⁵). The adduct of X^- with a Z -nucleus acts as a superheavy isotope of $(Z-1)$. It is shown here that although their geochemical separation would be less obvious than that of fractionally charged species containing unsaturated quarks^{6,7} it should still be possible to concentrate these exotic isotopes. Exceptional cases are the neutral adducts pX and 2DX , which may form tiny molecules with a second proton, or a polymer with a density $M \times 10^{10} \text{ g cm}^{-3}$ which could act as a low-temperature fusion catalyst and explain the excess heat irradiated by Jupiter.

The fusion of four protons to ^4He in most stars (including our Sun with a central temperature close to $15 \times 10^6 \text{ K}$) does not go⁴ mainly via the C–N–O catalysis proposed by Bethe, but rather takes place along a more direct path starting with two protons forming a deuteron, a positron and a neutrino^{8,9}. Zweig¹⁰ pointed out that unusual quarks with charge $(-4e/3)$ could bring together two deuterons, providing a one-stage fusion catalysis, if the quark has a much longer lifetime than the negative muon known¹¹ to promote fusion in liquid hydrogen. If the stellar interior contains a small concentration of X^- which does not become inactivated by trapping on unreactive nuclei, the neutral pX starts by capturing a second proton to form a 'molecule' pXp^+ having a wave function analogous to the hydride anion containing two electrons with a binding energy 1,836 times the electron affinity 0.75 eV of the hydrogen atom, not much above the prevailing kT . However, its ephemeral (but repeated) existence may help the otherwise low rate of forming 2DX and the two leptons. Although Coulomb barriers could be entirely avoided by subsequent reactions ($pX + ^3\text{He}$) or ($^2DX + ^2\text{D}$) with increasing cross-sections at lower temperature due to longer DeBroglie wavelengths, the major problem in stellar interiors is the trapping of X^- on helium, carbon and so on. The non-relativistic Schrödinger energy for the ground state of a system consisting of X^- and a nucleus with atomic weight A and charge Ze (where 1,823 is the reciprocal atomic weight of the electron) is

$$-AZ^2 \cdot 1,823 \cdot 13.6 \text{ eV} \quad (1)$$

almost independent of the atomic weight M of X^- (if $M \gg A$ the nucleus becomes the 'easily mobile' particle). For pX , equation (1) gives the binding energy as 25 keV, about 20 times kT at the solar centre, whereas $^3\text{He}X$ would have 300 keV and $^{12}\text{C}X$ 10.8 MeV, although the spatial extension⁶ of X^- and ^{12}C would decrease the latter value⁵. Exotic lithium may contain⁵ not only $^9\text{Be}X$ but also $^8\text{Be}X$ (where the X^- prevents the almost instantaneous fission in two α -particles) removing the bottleneck⁸ of nucleosynthesis.

Jupiter has an unaccounted heat production¹² slightly above $10^{24} \text{ erg s}^{-1}$, which is of the same order of magnitude as the insolation (with an albedo 0.42). As the mass of Jupiter is $1.9 \times 10^{30} \text{ g}$, this corresponds to an average energy production of $6 \times 10^{-7} \text{ erg g}^{-1} \text{ s}^{-1}$ in comparison with $1.87 \text{ erg g}^{-1} \text{ s}^{-1}$ for solar material. The temperature (128 K) doubles¹³ when looking down in the atmosphere through the 'window' at $2,000 \text{ cm}^{-1}$ where the IR lines are weak. If this excess heat production had continued at the present rate for $3 \times 10^9 \text{ yr}$, then 10^{-8} of the Jupiter mass would have had to be transmuted from hydrogen to helium. A simpler alternative would be that the excess heat was due to the gravitational energy released by radial contraction. Indeed, the same amount of heat could be evolved by the radius decreasing $\sim 400 \text{ km}$ or 1%. But although such a contraction may seem insignificant, this slow re-adaptation of the radius causes several problems, one being that if there was a time when the temperature was double its present value, the radiative cooling would have been 16 times more rapid.

Although the rotational ground state of pX does not have an electric dipole moment (if X^- is a fermion, the hyperfine splitting between $I = 0$ and 1 may be several eV) it is important to know whether pX (having the average distance $3 \text{ bohr}/2 \times 1,836 = 4.3 \times 10^{-12} \text{ cm}$) dimerizes, or polymerizes at room temperature to a highly compact liquid. The electrostatic dimerization energy of pX is ~ 10 –15 keV. If droplets of such an exotic liquid exist (with a density close to M times $10^{10} \text{ g cm}^{-3}$) they would form a general catalyst for nuclear reactions, and because of $M \gg 1$, they lose X^- by evaporation much less readily than protons. Such a material would be rather similar to a metal which has had its conduction electrons replaced by protons.

At first, one would expect irreversible trapping of X^- in CH_4 , NH_3 , and so on to create a worse problem in Jupiter than in the Sun, but the core of Jupiter is under such strong pressure that it may contain huge volumes of metallic hydrogen fairly separate from gaseous helium, and from the heavier elements, which may

not be soluble in metallic hydrogen. On the other hand, pX is expected to be soluble, and even effectively extracted. No fusion process is observed in hydrogen bubble chambers but all terrestrial hydrogen may have been stripped of X^- in early stages of the formation of Earth. An experiment at the Rutherford laboratory¹⁴ has put extremely low limits (10^{-29} – 10^{-30} X per proton if M is between 20 and 1,400). Direct mass spectrometric higher limits are close to 10^{-22} . Unfortunately, no comparable data have been presented for M between 10^4 and 10^6 .

The relative concentration of exotic isotopes of a given element Z would be expected to be proportional to the ratio between the abundances of $(Z+1)$ and of Z . Exotic boron, scandium, manganese and thallium would be good candidates from this point of view⁵. As boron isotopes ^{10}B and ^{11}B can be separated on a commercial scale it might be useful to attempt to detect ^{12}CX in the extreme fractions, although most boric acid has already been evaporated once from volcanoes. On the other hand, isotopes with A even as high as 10^6 should not be strongly fractionated in amorphous or crystalline solids by terrestrial gravitation, again except for polymeric pX . The latter should behave in a similar manner to that predicted¹⁵ for high- M magnetic monopoles concentrating at the centre of the Earth. As technetium and promethium possess only β -unstable isotopes, it might be useful to look for them in minerals as adducts of X^- and ruthenium or samarium. In concentrated uranium minerals, spontaneous and neutron-induced fission maintain¹⁶ concentrations of 10^{-17} promethium and 10^{-12} (of the longer lived) technetium. The search has only^{16,17} been for the specific isotope ^{147}Pm but it would be interesting to know whether it is accompanied by comparable amounts of SmX .

Cahn and Glashow⁵ point out that the presence of X^- decreases the energy of α -decay to the extent that ^{244}PuX and ^{247}CmX may be long-lived on a geochemical time scale. Such exotic isotopes would show up as the (ultramicroscopic amount) neptunium and (as yet unreported) americium in minerals. These authors do not contemplate the capture of X^- by the heavy nuclei, which would be likely to induce fission, but consider the buildup of 8BeX (not representing a barrier to nucleosynthesis⁸ like the instantaneous decay of 8Be) by adding many consecutive 4He . Such a process may have occurred shortly after the Big Bang and give products normally only obtained⁸ in supernova explosions. One of the attractive candidates would be exotic actinium ^{232}ThX . Because of radioactive equilibria, there are 10^{-15} actinium, 10^{-12} protactinium (slightly below the concentration of ^{226}Ra) to be compared with 12 p.p.m. of thorium, 16 p.p.m. of lead and 4 p.p.m. of uranium in the Earth's crust. If actinium is separated from a thorium mineral containing $<0.3\%$ uranium, the amount should be $<10^{-12}$ of the thorium. If a percentage of such a sample were ^{232}ThX rather than ^{227}Ac , it would establish a concentration of 10^{-14} X^- per nucleus or 5×10^{-17} X^- per nucleon. The same argument would apply to a rare-earth mineral (containing $<0.3\%$ uranium) where exotic actinium may be more likely to be situated by the geochemical fractionation. These limits are far lower than the 10^{-10} first suggested¹ and not much higher than the ratio between unsaturated quarks and nucleons, which is likely^{6,18} to have an order of magnitude of 10^{-20} (6,000 per g) if the existence of such species is not excluded by dogmatic quark confinement. It is possible that the very high atomic weights of exotic isotopes are concentrated on definite spots in large-scale mass spectrometers (like Oak Ridge calutrons) but there has been little effort to find them. A tandem accelerator¹⁹ was recently used to detect oxygen isotopes with (not necessarily integral) atomic weights between 20 and 54, and upper limits for abundances 10^{-16} – 10^{-18} were found. However, the exotic ^{19}FX discussed here would be heavier, and not particularly abundant⁸. A few atoms of ^{235}UX may be detected by irradiating a photographic plate (or transparent plastic), having been exposed to the ultra-high M -region of the mass spectrum of protactinium, with slow neutrons at high flux inducing fission. In such an experiment, the beam of Pa^+ should be as free of neutral atoms (and molecules) as possible. The asymmetry of the short and intense track of the

fragment carrying X^- and the long track of the other, much more rapid, fragment should be conspicuous.

If positive X^+ can be bound to nuclei (as predicted⁷ for positive quarks) by short-range interactions compensating for the electrostatic repulsion, separation of lithium would become of major interest as it would contain the adduct of 4He with X^+ ; and the exceedingly low concentrations of polonium and of neptunium in minerals might contain the adducts of ^{209}Bi and ^{238}U with X^+ .

The two most promising concentration techniques seem to be ultracentrifugation and fractional distillation of liquid air. In aqueous, quite concentrated solutions, ultracentrifugation is an excellent way of separating proteins or synthetic polymer molecules according to molecular weight. Supposing kilogramme quantities of dissolved lithium chloride were suspected of containing traces of 8BeX or 9BeX , then by adding a separation buffer, such as gramme quantities of caesium chloride, any exotic lithium would show up in the almost vanishing, heavier fraction. On the other hand, if caesium nitrate is suspected of containing adducts of X^- with barium, $N(C_4H_9)_4NO_3^-$ or thallium nitrate may be used as separating agents.

The technology of separating the constituents of atmospheric air is highly developed. The boiling points of 3He and 4He are sufficiently different that 6LiX may boil at a higher temperature than neon. There is an enormous gap between the boiling point 27 K of the (18 p.p.m. of volume) neon and 77 K of the major constituent N_2 without any known, or conceivable, minor constituent. Exotic neon ^{23}NaX would be situated in this gap. There is much less neon in our atmosphere than extrapolated from the cosmic abundance⁸ and a preliminary concentration by a factor of several thousand is obtained by the Earth having a gravitational field strong enough to retain exotic (but not conventional) neon over 10^9 yr.

Holt *et al.*²⁰ wanted to investigate a possible superdense modification of nuclear matter by looking for the noble gas radon (of which the longest living isotope known ^{222}Rn has the half-life 3.8 days) in xenon fractions from liquid air. Their negative result (with the higher limit 10^{-16} atom per xenon atom) is relevant to our problem. Because francium is even less abundant than radon, the search was really for exotic xenon formed by ^{133}CsX , and caesium has a rather low cosmic abundance. Gaseous diffusion or centrifugation of krypton, which contains²¹ 0.01% CF_4 , or collecting the almost absent fractions between krypton and xenon might enrich exotic krypton formed from the more abundant rubidium.

As pointed out by Fairbank⁶, exotic isotopes might be detected in dilute gaseous systems by the laser techniques developed²² for detecting single atoms. An especially clear case is the atomic spectrum of lithium, where the $2p \rightarrow 2s$ transition in the red has 0.2 cm^{-1} lower wavenumbers in 6Li than in 7Li . If this isotope shift is exclusively an effect of the electron moving the centre of gravity, it is 42 times smaller than the shift expected for any heavy isotope and the line positions then hardly depend on the explicit M value. Also exotic rubidium and caesium atoms should be quite good candidates for accurate monochromatic laser excitation but here the isotope shift is not due to a modified Rydberg constant. For our purposes, it is important that single xenon atoms²³ have been detected.

I thank Professor Sheldon L. Glashow, for suggestions and fruitful discussions. I also thank him and Dr Robert N. Cahn for the important preprint (ref. 5), and Professor Renata Reisfeld for valuable discussions over indefinite continual stratification in matter.

Received 11 February; accepted 27 April 1981.

1. Dover, C. B., Gaisser, T. K. & Steigman, G. *Phys. Rev. Lett.* **42**, 1117–1120 (1979).
2. Farhi, E. & Susskind, L. *Phys. Rev. D* **20**, 3404–3411 (1979).
3. Eichten, E. & Lane, K. D. *Phys. Lett.* **90B**, 125–130 (1980).
4. Rajpoot, S. *Phys. Rev. D* **22**, 2244–2254 (1980).
5. Cahn, R. N. & Glashow, S. L. *Chemical Signatures for Superheavy Elementary Particles*, LBL-12010 (Berkeley, 1980).
6. Jørgensen, C. K. *Struct. Bond.* **34**, 19–38 (1978); **43**, 1–36 (1981).
7. De Rújula, A., Giles, R. C. & Jaffe, R. L. *Phys. Rev. D* **17**, 285–301 (1978).
8. Trimble, V. *Rev. mod. Phys.* **47**, 877–976 (1975).
9. Kuchowicz, B. *Rep. Prog. Phys.* **39**, 291–343 (1976).

10. Zweig, G. *Science* **201**, 973–979 (1978).
11. Alvarez, L. W. et al. *Phys. Rev.* **105**, 1127–1128 (1957).
12. Chase, S. C. et al. *Science* **183**, 315–317 (1974).
13. Terrile, R. J. et al. *Science* **204**, 1007–1008 (1979).
14. *CERN Courier*, 18–21 (January 1981).
15. Carrigan, R. A. *Nature* **288**, 348–350 (1980).
16. Kenna, B. T. & Attrep, M. *J. inorg. nucl. Chem.* **28**, 1491–1500 (1966).
17. Attrep, M. & Kuroda, P. K. *J. inorg. nucl. Chem.* **30**, 699–703 (1968).
18. Wagoner, R. V. & Steigman, G. *Phys. Rev.* **D20**, 825–829 (1979).
19. Middleton, R. et al. *Phys. Rev. Lett.* **43**, 429–431 (1979).
20. Holt, R. J. et al. *Phys. Rev. Lett.* **36**, 183–186 (1976).
21. Gassmann, M. *Naturwissenschaften* **61**, 127 (1974).
22. Hurst, G. S. et al. *Rev. mod. Phys.* **51**, 767–819 (1979).
23. Chen, C. H., Hurst, G. S. & Payne, M. G. *Chem. phys. Lett.* **75**, 473–477 (1980).

²⁴¹Am from the decay of ²⁴¹Pu in the Irish Sea

J. P. Day & J. E. Cross

Department of Chemistry, University of Manchester, Manchester M13 9PL, UK

Americium-241 is a long-lived α -emitting nuclide which has become one of the more important marine contaminants arising from the discharge of effluents to the Irish Sea from the nuclear fuel reprocessing plant at Windscale, Cumbria^{1–3}. These discharges contain ²⁴¹Am, currently ~ 200 Ci yr⁻¹, and also the β -emitting nuclide, ²⁴¹Pu (half life 14.7 yr), which decays in the environment to ²⁴¹Am. Both americium and plutonium seem to be largely retained in sediments relatively close to Windscale^{4–7}, and a reservoir of ²⁴¹Am is building up both from direct deposition and from the radioactive decay of sedimented ²⁴¹Pu. Here we attempt to estimate quantitatively the size and rate of growth of this reservoir. Using data for Windscale discharges^{1–4}, we have estimated the probable sedimentary deposition of ²⁴¹Am and ²⁴¹Pu over the past 20 yr, and calculated the consequent additional ingrowth of ²⁴¹Am. The latter is now ~ 600 Ci yr⁻¹, rising towards a steady state $\sim 1,300$ Ci yr⁻¹ if present rates of discharge of ²⁴¹Pu are maintained. The present sedimentary reservoir of ²⁴¹Am is $\sim 18,000$ Ci, of which about 4,800 have resulted from ²⁴¹Pu decay *in situ*. Measurement of the ²⁴¹Pu/²⁴¹Am isotope ratio in coastal sediments confirms our estimates of ²⁴¹Pu deposition. Outside the Irish Sea, in the dispersal plume into Scottish waters, we have concluded that the observed ²⁴¹Am concentration⁸ can be accounted for largely by ingrowth from the ²⁴¹Pu transported in the water mass.

It has been established^{4,5}, by measurement of the activities of the α emitters ²³⁸Pu and ²³⁹⁺²⁴⁰Pu, in Irish Sea sediments, that >95% of the plutonium released from Windscale during the past 20–25 yr has collected relatively close to the point of discharge. In many locations, these sediments have remained largely undisturbed and the sedimentary record with respect to several isotopes, such as plutonium^{4,5,9}, caesium and other nuclides¹⁰ can be related satisfactorily to the history of the Windscale discharges. Although levels of ²⁴¹Pu in sediments have not been measured systematically, the accumulation and subsequent decay of ²⁴¹Pu to ²⁴¹Am can readily be calculated if the quantities of ²⁴¹Pu initially deposited can be estimated.

It also seems likely that the marine discharges of ²⁴¹Am from Windscale will have largely accumulated in sediments in the region, although this possibility has not yet been established from measurement of activity levels in cores. The relative adsorption of ²⁴¹Am by suspended particulates in seawater in the Windscale area has been found^{6,7} to be greater than for ²³⁹Pu, and the settling out of such material would, therefore, be expected to remove americium more rapidly than plutonium. Consequently, almost the entire inventory of ²⁴¹Am, both discharged and ingrown, for the 20–25 yr of plant operation will probably be present in the sediments of the region. Interpretation of the sedimentary record for ²⁴¹Am can only be achieved if due allowance is made for ²⁴¹Pu deposition and decay.

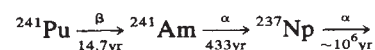
Table 1 Marine discharges from Windscale and environmental accumulation of ²⁴¹Pu and consequent production of ²⁴¹Am

Year	Windscale discharges (Ci yr ⁻¹)			Accumulated ²⁴¹ Pu (Ci)	Production of ²⁴¹ Am (Ci yr ⁻¹)
	Pu- α	²⁴¹ Pu	²⁴¹ Am		
1960	78	2,255	—	2,203	2
1961	104	3,012	—	5,044	6
1962	186	5,413	—	10,099	12
1963	233	6,780	—	16,257	21
1964	285	8,294	—	23,610	32
1965	292	8,497	—	30,823	44
1966	292	8,497	—	37,704	55
1967	499	14,521	—	50,152	70
1968	828	24,095	576	71,379	97
1969	816	23,920	396	91,458	130
1970	936	27,092	540	113,711	164
1971	1,128	32,970	1,020	140,680	204
1972	1,548	45,163	2,172	178,319	255
1973	1,776	51,944	2,952	220,847	320
1974	1,248	36,491	3,192	246,322	374
1975	1,257	36,579	1,052	270,708	414
1976	1,266	35,048	323	292,477	451
1977	981	26,517	99	304,909	478
1978	1,567	47,928	214	337,684	514
1979	1,335	40,383	212	361,579	560
1980	—	—	—	381,527	595

Pu- α includes ²³⁸+²³⁹+²⁴⁰Pu (refs 1, 2, 4). ²⁴¹Am data are not available before 1968, although discharges presumably occurred. ²⁴¹Pu data were not published before 1976; we have estimated these releases (shown in italics) from the Pu- α activities, using the mean ²⁴¹Pu/Pu- α isotope ratio (29.1) between 1976 and 1979. The environmental accumulation of ²⁴¹Pu, and consequent production of ²⁴¹Am, resulting from the Windscale discharges and corrected for radioactive decay were calculated by numerical approximation, using equations (1) and (2) and computed on a daily basis (assuming 1/365 of the annual discharge for each day). For calculation, the annual discharge of ²⁴¹Pu subsequent to 1979 has been taken as 37,469 Ci yr⁻¹, the average for the years 1976–79.

The potential radiological importance of ²⁴¹Am, which reaches human consumption largely through fish and shellfish, became particularly apparent¹ in about 1975–76, following several years of relatively large discharges of ²⁴¹Am from Windscale (Table 1). The relative contributions of discharged and ingrown ²⁴¹Am to the environmental pool available to marine organisms has been examined¹¹, and the potential radiological significance of sediment held ²⁴¹Am is thought to be small^{11,12}. However, the conditions in which americium could be remobilized from sediments have not yet been fully investigated.

The ingrowth of ²⁴¹Am in sediments has been calculated from the estimated discharges of ²⁴¹Pu (Table 1), assuming total deposition of discharged plutonium. Plutonium-241 decays as follows:



The quantities of ²⁴¹Am formed and ²⁴¹Pu remaining, following the decay of a given initial quantity of ²⁴¹Pu, are given by equations (1) and (2):

$$[^{241}\text{Am}]_t = [^{241}\text{Pu}]_0 \frac{T_{\text{Pu}}}{(T_{\text{Am}} - T_{\text{Pu}})} \exp\left(-\frac{t \ln 2}{T_{\text{Am}}}\right) - \exp\left(-\frac{t \ln 2}{T_{\text{Pu}}}\right) \quad (1)$$

$$[^{241}\text{Pu}]_t = [^{241}\text{Pu}]_0 \exp\left(-\frac{t \ln 2}{T_{\text{Pu}}}\right) \quad (2)$$

where T_{Pu} and T_{Am} are the radioactive half lives of ²⁴¹Pu and ²⁴¹Am, respectively.

The sedimentary accumulation of ²⁴¹Pu by the end of each year, and the corresponding annual ingrowth of ²⁴¹Am, is given in Table 1. The rate of generation of ²⁴¹Am has increased steadily, and by 1980 had reached a level ~ 600 Ci yr⁻¹. The continued discharge of ²⁴¹Pu at a constant annual rate would eventually give rise to a steady-state condition in the sediments in which the rates of deposition, and loss by radioactive decay, would have become equal. The steady state would be

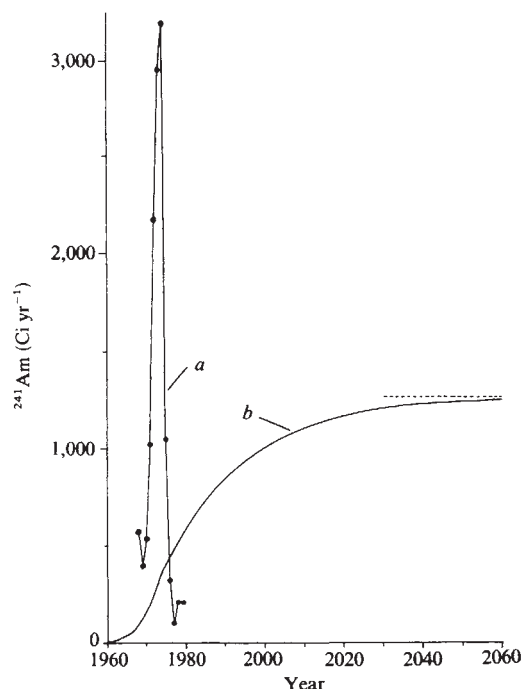


Fig. 1 Input of ^{241}Am to the environment. *a*, Direct discharge from Windscale; *b*, calculated production from radioactive decay of accumulated ^{241}Pu in the environment from Windscale discharges.

approached exponentially (Fig. 1), with a half time of approach equal to the decay half life of ^{241}Pu . Assuming that discharges of ^{241}Pu remain at about their present levels, the ingrowth of ^{241}Am will level off at about $\sim 1,300 \text{ Ci yr}^{-1}$. It is, of course, unlikely that discharges from Windscale will stay constant for this period in the future. However, the relative importance of the present rates of discharge of ^{241}Pu and ^{241}Am to future levels of ^{241}Am in the sedimentary environment is clear.

The sedimentary reservoir of ^{241}Am in the Irish Sea can be estimated if it is assumed (as seems likely) that effectively all the available americium, whether discharged from Windscale or ingrown, has been retained in the sediments. For the period 1960–80 we estimate that $\sim 4,800 \text{ Ci}$ of ^{241}Am will have ingrown from ^{241}Pu , whilst $\sim 13,000 \text{ Ci}$ has originated from direct deposition (this does not include any ^{241}Am discharged before 1968, for which we have no data, but it seems likely that the total released up to 1968 would have been relatively small, perhaps $\sim 1,000 \text{ Ci}$). Correction for decay over this time period is insignificant, and the total reservoir is therefore $\sim 18,000 \text{ Ci}$.

To test these conclusions, we have measured the overall $^{241}\text{Pu}/\text{Pu-}\alpha$ isotope ratio in sediments taken from the Ravensglass Estuary, adjacent to Windscale. Two cores, taken in 1978 to a depth sufficient to include the deposition for at least 20 yr, were homogenized and sub-samples analysed radiochemically. Plutonium was separated from the material by procedures^{13,14} which exclude americium, and the activities of ^{238}Pu , ^{239}Pu and ^{241}Am determined by α spectrometry, both initially ($^{241}\text{Am} = 0$) and after about 14 months (Table 2). The measurement of ingrown ^{241}Am allowed the activity of the ^{241}Pu present in the original sample to be calculated¹⁵.

The $^{241}\text{Pu}/\text{Pu-}\alpha$ activity ratio (23.1) determined in these sediments is very close to that calculated (22.0) by summing the $\text{Pu-}\alpha$ and ^{241}Pu discharges (corrected for decay) up to the end of 1978. This value is significantly different from the current ratio at discharge, for which $^{241}\text{Pu}/\text{Pu-}\alpha = 29.1$ (average, 1976–79). The close agreement between the measured and calculated values we take to confirm both that continuous accumulation of plutonium in sediments has occurred over that time, and also that our estimates of ^{241}Pu discharges for the years 1960–75 (Table 1) are essentially correct.

As an extension of these calculations, we have re-examined the data obtained by Murray *et al.*⁸ for the activities of ^{241}Am , ^{238}Pu and $^{239+240}\text{Pu}$ in Scottish coastal waters. It has been established^{16,17}, largely from ^{134}Cs and ^{137}Cs measurements, that the main dispersal plume of radioactivity from the Irish Sea emerges northwards, passes along the west and north coasts of Scotland, and in part reaches the North Sea. The approximate average transit times for nuclides (1) to emerge from the North Channel of the Irish Sea¹⁶, and (2) to reach the sea area off the north-west corner of Scotland¹⁷ (Cape Wrath), following discharges from Windscale, are estimated at ~ 1.5 and 2 yr, respectively. The activity ratio, $^{241}\text{Am}/\text{Pu-}\alpha$, decreases from ~ 1.7 at discharge to ~ 0.06 in waters to the west and north of Scotland, as observed by Murray *et al.*⁸ who concluded that the ^{241}Am and $\text{Pu-}\alpha$ are both of direct Windscale origin, the americium having been more rapidly removed from the water column. However, although qualitative reference was made^{8,17} to the possible contribution of ^{241}Am ingrown from ^{241}Pu , this was not estimated quantitatively.

We now suggest that these data, at least for the more northerly Scottish waters, can be interpreted on the hypothesis that the ^{241}Am arises almost entirely by ingrowth from ^{241}Pu during transport of the water mass. For the sea area to the north-west of Cape Wrath, the mean activities of ^{241}Am and $^{238+239+240}\text{Pu}$ ($\text{Pu-}\alpha$) from Murray *et al.*⁸ were 0.097 and 1.60 fCi l^{-1} , respectively. The inferred ^{241}Pu activity, $\sim 44 \text{ fCi l}^{-1}$, would be sufficient to generate the observed ^{241}Am concentration over a period of ~ 1.4 yr. This is somewhat longer than the estimated transit time from the North Channel of the Irish Sea, but significantly less than the transit time from Windscale. If we assume that the ^{241}Am concentration results from an approximate steady-state condition, between rate of loss from the water mass (for example, by sedimentation) and rate of replacement by ingrowth, then the replacement time previously calculated (~ 1.4 yr) can be identified as the approximate mean residence time¹⁸ for ^{241}Am in these waters. The probable existence of such a steady state is supported by the approximately constant value (between 0.05 and 0.08) for the $^{241}\text{Am}/\text{Pu-}\alpha$ activity ratio round the Scottish coastline from the Minch to the North Sea⁸.

Although these conclusions are based primarily on the decay characteristics and observed activities in seawater of the plutonium and americium nuclides, the conclusions are not inconsistent with other facets of the marine behaviour of these elements, and may even help to remove an apparent anomaly. Thus, whilst most of the Windscale-discharged plutonium and americium seems to be rapidly removed to sediments, the remainder seems to be relatively well conserved in seawater, and to follow ^{137}Cs in the dispersal plume. For plutonium, the probable explanation is that the sedimented and conserved fractions correspond to the element in the two different (environmentally stable) oxidation states, Pu(IV) and Pu(VI) , respectively^{19,20}. For americium, the only plausible oxidation state in solution is Am(III) , which is firmly and rapidly retained by sedimentary material within the Irish Sea^{6,7}. Thus, the

Table 2 Activities of ^{238}Pu , ^{239}Pu and ingrown ^{241}Am from sediment cores collected near Windscale at the end of 1978

Nuclide	Source activities (pCi)	
	Sample 1	Sample 2
^{238}Pu	25.3	26.7
^{239}Pu	102.2	107.7
^{241}Am	5.46	5.73
^{241}Pu	2,953	3,099
Ratio $^{241}\text{Pu}/\text{Pu-}\alpha$	23.2	23.1

Activities were determined by α spectrometry 434 days after the preparation of two sources from sediment cores collected from Ravensglass Estuary. The cores ($\sim 50 \text{ cm}$) correspond to a sedimentary deposition covering at least 20 yr, determined (J.E.C., unpublished) from the $^{134}\text{Cs}/^{137}\text{Cs}$ and $^{238}\text{Pu}/^{239}\text{Pu}$ isotope ratios^{4,9,10}. The ^{241}Pu activities were calculated¹⁵ from ingrown ^{241}Am . The measured activity ratio, $^{241}\text{Pu}/\text{Pu-}\alpha = 23.1$, is very similar to that calculated by summation of the discharges up to 1978 (Table 1) that is $^{241}\text{Pu}/\text{Pu-}\alpha = 22.04$.

apparent maintenance of a rather constant Am/Pu ratio in Scottish waters is difficult to explain in chemical terms, as at first sight the phenomenon seems to imply a conserved fraction for americium, as well as for plutonium, in this water. However, we think a possible explanation, in terms of ingrowth from ^{241}Pu coupled with a rather short residence time for ^{241}Am in the water phase, is more plausible and is compatible both with the available environmental data and with the present understanding of the marine chemistry of americium.

We thank the SRC for the award of a research Studentship to J.E.C.

Received 16 December 1980; accepted 1 May 1981.

- Hunt, G. J. *Radioactivity in Surface and Coastal Waters of the British Isles, 1978* (Directorate of Fisheries Research, Ministry of Agriculture, Fisheries and Food, Lowestoft, 1979).
- Annual Survey of Radioactive Discharges in Great Britain, 1979* (Department of the Environment, London, 1980).
- Atherton, R. S. *Annual Report of Radioactive Discharges and Monitoring of the Environment, 1979* (British Nuclear Fuels, Health and Safety Directorate, Risley, Warrington, 1978).
- Hetherington, J. A. in *Environmental Toxicity of Aquatic Radionuclides: Models and Mechanisms* (eds Miller, M. W. & Stannard, J. N.) Ch. 5 (Ann Arbor, Michigan, 1976).
- Hetherington, J. A. *Mar. Sci. Commun.* **4**, 239–274 (1978).
- Hetherington, J. A. & Harvey, B. R. *Mar. Pollut. Bull.* **9**, 102–106 (1978).
- Pentreath, R. J., Jefferies, D. F., Lovett, M. B. & Nelson, D. M. *Proc. 3rd NEA Seminar on Marine Radioecology*, 203–221 (Nuclear Energy Agency, OECD, Tokyo, 1979).
- Murray, C. N., Kautsky, H., Hoppenheit, M., & Domian, M. *Nature* **276**, 225–230 (1978).
- Aston, S. R. & Stanners, D. A. *Nature* **289**, 581–582 (1981).
- Aston, S. R. & Stanners, D. A. *Estuar. coast. mar. Sci.* **9**, 529–541 (1979).
- Pentreath, R. J. *Int. Symp. on the Impacts of Radionuclide Releases into the Marine Environment* (IAEA, Vienna, 1980).
- Hunt, G. J. & Jefferies, D. F. *Int. Symp. on the Impacts of Radionuclide Releases into the Marine Environment* (IAEA, Vienna, 1980).
- Hampson, B. L. & Tennant, D. *Analyst* **98**, 873–885 (1973).
- Cross, J. E. & Day, J. P. *Envir. Pollut. B* **2**, 249–257 (1981).
- Livingston, H. D., Schneider, D. L. & Bowen, V. T. *Earth planet. Sci. Lett.* **25**, 361–367 (1975).
- Jefferies, D. F., Preston, A. & Steele, A. K. *Mar. Pollut. Bull.* **4**, 118–122 (1973).
- Livingston, H. D. & Bowen, V. T. *Nature* **269**, 586–588 (1977).
- Holland, H. D. *The Chemistry of the Atmosphere of the Atmosphere and Oceans*, 5 (Wiley-Interscience, New York, 1978).
- Nelson, D. M. & Lovett, M. B. *Nature* **276**, 599–601 (1978).
- Aston, S. R. *Mar. Chem.* **8**, 319–325 (1980).

Binary mixing in ocean-ridge spreading segments

M. F. J. Flower

Department of Geological Sciences, University of Illinois, Chicago, Illinois 60680, USA

Compositional mixing trends in eruptive mid-ocean ridge basalt (MORB) seem to be confined to short time-space intervals, possibly corresponding to single fracture-zone-bounded segments of a spreading ridge axis. Binary mixing is discernible from the variation in Ta, Hf, Rb, Sr and rare earth elements (REEs), and may be interpreted as reflecting dual mantle sources for locally-defined units of the axis or associated transform fracture zones. These sources reflect contrasting degrees and types of incompatible (or 'low- K_D ') element enrichment relative to a 'normal' MORB source characterized by uniform depletion in these elements. Enrichment in low- K_D elements, radiogenic isotopes and normative nepheline has long been recognized in some fracture zone magmas^{1–4} and it is important to speculate on the possible influence of fracture zones on the chemical character of spreading axes. Consideration of chemical and isotopic variation at a single Atlantic spreading segment (36–37°N) in a regional context suggests that transform fracture zones may be instrumental in both generating compositional heterogeneity in the mantle and permitting its expression in eruptive crust. It is proposed here that hybridization of 'enriched' fracture zone-derived melt and less-enriched or depleted rift-derived melt occurs in sub-axial magma supply systems during an active spreading phase.

Between the Azores Plateau (40°N) and ~30°N the Mid-Atlantic Ridge (MAR) is 'transitional' in type and characterized by short offset spreading segments forming a SSW-trending zone of accretion. FAMOUS and AMAR studies of the axial region at 36–37°N (refs 5–7) documented geophysical and petrological properties of such segments (Rift Valleys (RV) 2 and 3) and DSDP Leg 37⁸ studied their stratigraphic expression at four sites (332–335) perpendicular to the ridge axis. The sites represent a spreading flow line between 3.4 and 13 Myr BP. Most DSDP/IPOD basement sites reflect negligible source-related chemical differences between stratigraphically contiguous lava series⁹. In contrast, the source-related chemical diversity at Site 332 and RV-2 (refs 10–12) is apparently characteristic of transitional ridge axes^{2,4}, and precludes common derivation for these series through processes of fractional crystallization in small reservoir systems^{10–12}.

While some interpretations of 36–37°N have failed to emphasize the transitional character of the ridge axis, Schilling *et al.*¹³ explained REE variation in this region in the context of spatial and temporal trends, postulating influx of an Azores plume or mantle 'blob'¹⁴. However, the case for binary mixing of sources on the scale required by this model, at least in simple form, is not supported by regional isotope and low- K_D element variation for either secular (0–13 Myr) or isochronic (zero age) trends¹⁵. Other explanations for anomalous geochemistry in 36–37°N magmas appealed to zoned and/or periodically refilled magma chambers^{16,17}, and also to the process of 'dynamic melting'¹⁰ which involves incremental melting and incomplete extraction of the melt phase as a typical feature of dilating partial melting environments. These concepts have been questioned¹⁸, and in the absence of seismic recognition and petrological evidence for sub-rift reservoirs^{19–21} and the lack of chemical characteristics ascribed to dynamic melting at 'normal' ridge axes (for example, 22°N), it is reasonable to explore alternative models.

Evidence from lava and xenolith variation at single (such as island) localities suggests that mantle heterogeneity is possibly vertical in nature^{4,22,23} thus coalescence of dilating and transform fractures could allow differential tapping of the mantle. Thermal models^{24,25} predict progressively shallower partial melting with increasing spreading rates. A simplistic but reasonable synthesis could postulate preferential zones of partial melting, activated by either normal rift dilation (high degrees of partial melting at shallow depth), or (depending on

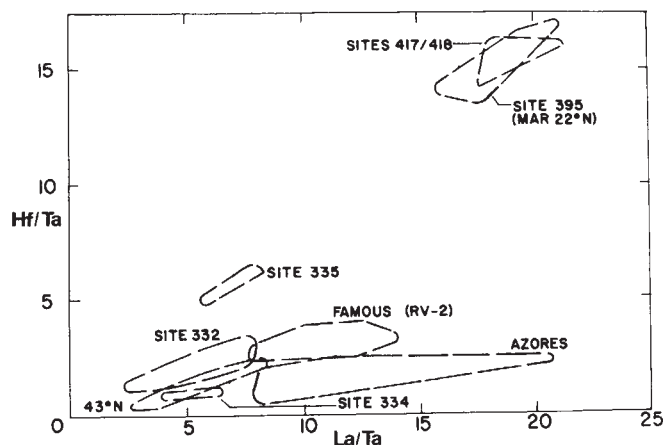


Fig. 1 Hf/Ta versus La/Ta diagram for basalts from the Atlantic/Azores region, data from refs 2, 8, 12, 36–39. Sites 395, 417 and 418 reflect 'normal' MORB depleted in low- K_D elements. Note the absence of regional binary mixing; the colinear trend of FAMOUS RV-2 and Site 332; the lack of such a trend between 332, 334 and 335 (36–37°N spreading flow line); and the diversity of 'enrichment' type compared with normal MORB.

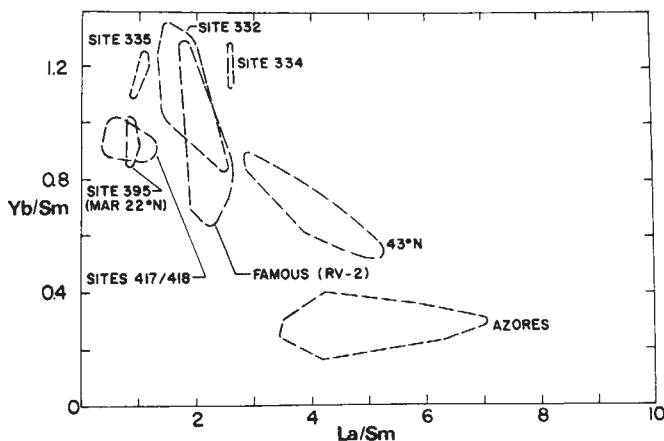


Fig. 2 Yb/Sm versus La/Sm diagram for basalts from the Atlantic/Azores region; data from same sources as for Fig. 1. Note the colinear RV-2-332 trend and lack of colinearity for 36–37°N flow line; and the greater distinction of La/Sm (lower K_D) than Yb/Sm (higher K_D).

ridge axis kinematics) by transform fractures (low degrees of partial melting at greater depth), each environment constrained by a different range of pressure, temperature and hence, depth of melting. In turn, a given fracture zone-bound spreading segment might be characterized by (1) relative volumetric contributions from the two environments, and (2) the extent to which these hybridize during storage and/or transport to the surface.

Considering the range of variation at 36–37°N it seems that a combination of all factors is required. MgO-rich basalts are depleted in light REEs¹⁰ and there is a general correlation (not attributable to fractional crystallization) of low- K_D element enrichment, increases in La/Sm, Rb/Sr, Ta/La and so on and decreasing Mg/(Mg + Fe²⁺). This correlation may reflect the combined effects of source heterogeneity and fractional crystallization, and probably also variations in the extent of partial melting, and as a general effect may indicate relatively direct access to the surface from the rift-melting environment, but more lengthy transport/storage (hence fractionation) histories for enriched magmas derived from fracture zone melting, before hybridization. Most mixing models have considered ridge-longitudinal 'open' feeder systems²⁷ which are probably not typical for slow-spreading axes such as the MAR²¹. Possible roles for fracture zones have not been seriously examined. If transform fractures permit the confluence of different magma supply systems, the development of chemical gradients at mid-ocean ridges may reflect the extent and nature of offset spreading as reflected by fracture zone frequency rather than regional mixing of different solid mantle fractions. This is apparent from recent data compilations²⁸ which show low- K_D element and radiogenic isotope peaks, together with lava temperature 'troughs', corresponding not only to positive gravity and thermal anomalies (proposed loci for mantle upwelling) but to major transform fractures. Mixing between fracture zone and rift systems is consistent with evidence for longitudinal magma flow at dilating ridges^{29,30} and with inferences from seismic and chemical data constraining magma systems to individual ridge segments^{11,19,20}.

Low- K_D element ratio-ratio plots are a useful way to study source-characteristic mixing. If each ratio denominator is identical (for example, K/Rb and Sr/Rb) mixing produces straight line variation between the two³¹. Data for DSDP Sites 332 (3.4 Myr), 334 (10 Myr) and 335 (13 Myr), FAMOUS RV-2, the Azores and other Atlantic localities are depicted in two low- K_D element ratio diagrams: Hf/Ta–La/Ta and Yb/Sm–La/Sm (Figs 1 and 2). Hf/Ta–La/Ta plots for RV-2 and 332 reflect significant variation in source composition, distinct from that of 'normal' ridge segments (represented by Sites 395, 417

and 418) and consistent with binary mixing. The variation is also distinct from that of older sites at 36–37°N and from other 'enriched' localities in the Atlantic. A similar, more diffuse, relationship is observed for Yb/Sm–La/Sm variation (Fig. 2). In general, variation for given localities such as spreading segments or individual islands is consistent with simple binary mixing, whereas variation on a regional scale reflects a complex array of fractionation processes in the mantle. The relation to small-scale rather than large-scale tectonic features is probably highly significant, and bears on fundamental problems concerning the chronology and origins of mantle heterogeneity.

⁸⁷Sr/⁸⁶Sr and ¹⁴³Nd/¹⁴⁴Nd ratios for FAMOUS RV-2 lavas¹⁸ indicate isotopic homogeneity for the source region at present, and, as for many low- K_D element-enriched magmas, reflect a time-integrated source depletion in Rb and LREE compared with chondrites^{18,32}. White¹⁸ suggests that this reflects that either Rb/Sr and Nd/Sm ratios in the source are uniform, or that heterogeneities in these ratios are young (≤ 80 Myr). Although White prefers the former interpretation, his data reflect Rb/Sr and Nd/Sm variation between 0.024–0.063 and 0.267–0.337 respectively (Fig. 3) and show a strong negative correlation. This clearly favours the interpretation that heterogeneity is of recent origin. The approximate coincidence of RV-2 and Site 332 Rb/Sr–Nd/Sm variation to bulk Earth estimates in Fig. 3 is probably fortuitous in view of the depletion history recorded by Sr and Nd isotopes. Together, the low- K_D and isotope components suggest that generation of heterogeneity expressed in the 36–37°N lavas is coeval with, and may be complementary to, processes of magma generation as determined by the overall tectonic environment. From geophysical considerations³³ local binary mixing of mantle fractions is unlikely, suggesting that mixing of different mantle-derived magmas is a more plausible mechanism for producing mantle-related mixing trends of the type observed. The fracture zone-rift explanation for binary mixing is also appealing for its ability to explain the origins of low- K_D element enrichment in the mantle. Enrichment by metasomatic veining of fertile peridotite has been documented in terms of mineralogy, chemistry and isotopic equilibration^{34,35} and may be the result of incipient melting in garnet lherzolite. The thermal-kinematic nature of transform fractures and the correspondence of magmatic enrichment/mixing trends to local rather than regional tectonic features suggests that transform fracturing may actually provoke

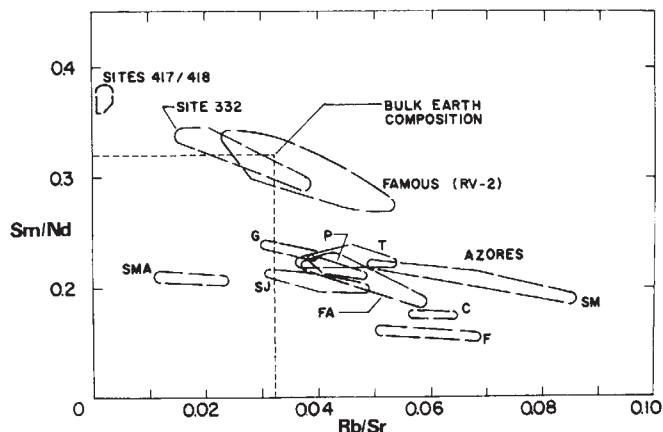


Fig. 3 Sm/Nd versus Rb/Sr diagram for basalts from the Atlantic/Azores region: data from refs 8, 10, 12, 18, 36–39. SMA, Santa Maria; G, Graciosa; T, Terceira; P, Pico; SJ, Sao Jorge; FA, Faial; C, Corvo; F, Flores and SM, Sao Miguel. Note the apparent correspondence of RV-2 and 332 to 'bulk Earth' composition (in contrast to Sr and Nd isotope compositions (ref. 18), 'depletion' of 'normal' MORB in Nd and Rb, and 'enrichment' of Azores in Rb (except Santa Maria) and Nd.

generation of low-percentage melt increments at depth, and provide upward access for these into depleted 'normal' mantle to form secondary 'enriched' sources for ridge axis magma. In this context, the evaluation of small variations in pressure, temperature, percent melting and disequilibrium in determining the nature of enrichment becomes critical in explaining the unique chemistries of enriched-magma associations.

I thank Paul Robinson, Hubert Staudigel and Alan Zindler for helpful discussions.

Received 6 January; accepted 27 April 1981.

1. Bonatti, E., Chermak, A. & Honnorez, J. *2nd Ewing Ser.* (eds. Talwani, M., Harrison, C. G. & Hayes, D. E.) (American Geophysical Union, 1979).
2. Shibata, T., Thompson, G. & Frey, F. A. *Contr. Miner. Petrol.* **70**, 127–141 (1979).
3. Bonatti, E. & Hamlyn, P. R. *Science* **201**, 249–251 (1978).
4. Batiza, R. & Johnson, J. R. *Init. Rep. DSDP 54* (in the press).
5. Needham, H. D. & Francheteau, J. *Earth planet. Sci. Lett.* **35**, 30–42 (1974).
6. Hekinian, R., Moore, J. G. & Bryan, W. B. *Contr. Miner. Petrol.* **58**, 83–110 (1976).
7. Ballard, R. D. *et al. EOS Union* **59**, 1198–1199 (1978).
8. Aumento, F. *et al. Init. Rep. DSDP Leg 37* (1977).
9. *Init. Rep. DSDP Legs 45, 46, 51–53* (1979–80).
10. Langmuir, C. H. *et al. Earth planet. Sci. Lett.* **36**, 133–156 (1978).
11. Flower, M. F. J. *et al. Contr. Miner. Petrol.* **64**, 167–195 (1977).
12. Bougault, H. *et al. Tectonophysics* **55**, 11–34 (1979).
13. Schilling, J. G., Kingsley, R. & Bergeron, M. *Init. Rep. DSDP Leg 37*, 591–598 (1977).
14. Schilling, J. G. *Earth planet. Sci. Lett.* **25**, 103–115 (1975).
15. Flower, M. F. J. *Lithos* (submitted).
16. Bryan, W. B., Thompson, G. & Michael, P. J. *Tectonophysics* **55**, 63–85 (1979).
17. O'Hara, M. J. *Nature* **266**, 503–507 (1977).
18. White, W. W. *Yb. Carnegie Instn Wash.* **78**, 325–331 (1979).
19. Fowler, C. M. R. *Geophys. J. R. astr. Soc.* **47**, 459–591 (1976).
20. Steinmetz, L., Whitmarsh, R. & Moreira, V. *Geophys. J. R. astr. Soc.* **50**, 353–380 (1977).
21. Flower, M. F. J. *Nature* **287**, 530–532 (1980).
22. Hofmann, A. W., White, W. M. & Whitford, D. J. *Yb. Carnegie Instn Wash.* **77**, 548–562 (1978).
23. Zindler, A. *et al. Earth planet. Sci. Lett.* **45**, 249–262 (1979).
24. Sleep, N. H. *J. geophys. Res.* **80**, 4037–4042 (1975).
25. Kuznir, N. J. & Bott, M. H. P. *Geophys. J. R. astr. Soc.* **47**, 83–95 (1976).
26. Bottinga, J. & Allègre C. J. *Phil. Trans. R. Soc. A288*, 501–525 (1979).
27. Rhodes, J. M. & Dungan, M. A. in *2nd Ewing Ser.* (eds Talwani, M., Harrison, C. G., Hayes, D. E.) 262–272 American Geophysical Union, 1979).
28. Schilling, J. G. & Sigurdson, H. *Nature* **282**, 370–375 (1980).
29. Vogt, P. R. *Earth planet. Sci. Lett.* **29**, 309–325 (1976).
30. Björnsson, A. *et al. Nature* **266**, 318–322 (1978).
31. Langmuir, C. H. *et al. Earth planet. Sci. Lett.* **37**, 380 (1978).
32. O'Nions, R. K., Hamilton, P. J. & Evensen, N. M. *Earth planet. Sci. Lett.* **34**, 13–32 (1977).
33. Forsyth, D. W. *Tectonophysics* **38**, 89–118 (1977).
34. Menzies, M. & Rama-Murthy, V. *Earth planet. Sci. Lett.* **46**, 323–334 (1980).
35. Wood, D. A. *Geology* **7**, 499–503 (1979).
36. Blanchard, D. P. *et al. J. geophys. Res.* **81**, 4231–4246 (1976).
37. White, W. M., Tapia, M. D. M. & Schilling, J. G. *Contr. Miner. Petrol.* **69**, 201–213 (1979).
38. Hawkesworth, C. *et al. Nature* **280**, 28–31 (1979).
39. Jahn, B. M. *et al. Earth planet. Sci. Lett.* **48**, 171–184 (1980).

Sanidine spherules at the Cretaceous–Tertiary boundary indicate a large impact event

J. Smit & G. Klaver

Geological Institute, Nieuwe Prinsengracht 130, Amsterdam, The Netherlands

The hypothesis that a catastrophic impact of an extraterrestrial body caused the terminal Cretaceous mass extinctions of dinosaurs, planktonic foraminifera and other species is now accepted as respectable following the discovery of a worldwide iridium enrichment in the Cretaceous–Tertiary (K–T) boundary clay^{1–5}. In the basal lamina of the K–T boundary clay of Caravaca (Spain)⁷ numerous spherules were discovered composed of finely crystallized, almost pure K-feldspar in the structural state of high sanidine. It is concluded here that these spherules solidified from a melt and were probably derived from the impacting body. This poses problems as high K-values are not reported from bulk analyses of meteorites⁶. The K-feldspar phenocrysts reported in some iron meteorites²³ suggest the body may have been a metal–sulphide–silicate planetesimal. A cometary body is suggested as an alternative.

The strongest geochemical extraterrestrial signals have been recorded in the basal lamina of the 10-cm thick K–T boundary clay of both the Barranco del Gredero section (Caravaca) and the 'Fish-clay' at Stevns Klint (Denmark)^{1–5}. These basal millimetres show the highest concentrations of the siderophile elements Ir (44 and 86.7 parts per 10⁹ respectively), Os, Cr, Co and Ni, and also a significant rare earth element (REE) depletion. (J.S. and Ten Kate, in preparation). The REE depletion, if caused by the low concentration of REE in the impacting body, suggests a large meteoritic contribution in the basal lamina, regarded as 'fall-out' level of the impact event. Moreover, anomalously high amounts of the chalcophile elements Zn, As, Se, and Sb have been found, elements which are not present in high concentrations in known extraterrestrial material⁶. The sanidine spherules occur as small (0.5–1 mm) globules, dumbbells and disks only in the 'fall-out' level of the K–T boundary clay at Caravaca; some 50–300 per cm³ sediment were found. X-ray diffraction analysis of the spherules has been carried out on a Guinier camera, using CuK α radiation and corundum as internal standard. The diffraction pattern shows only K-feldspar lines of good quality, which permit accurate determination of the 2θ angles (204; 50.78°, 060; 41.59°, 201; 20.98° ($\pm 0.1^\circ$)) and lattice constants (Table 1, Fig. 1). Indexing of the Guinier pattern according to Borg and Smith⁸ as sanidine is unambiguous.

Furthermore, the spherules were analysed chemically by electron microprobe and instrumental neutron activation analysis (INAA) (Table 1) and have been examined by scanning electron microscope and petrographic microscope (Figs 2, 3).

In good agreement with the X-ray diffraction, the microprobe analysis gives a K-feldspar composition of the formula $K_{0.95}Na_{0.01}Fe_{0.01}Al_{0.98}Si_{3.01}O_8$. The high K/Na ratio is remarkable: $K/(K+Na) = 0.99$. No difference in chemical composition has been observed between the centre and the edge of the spherules. Within the spherules occur numerous small opaque inclusions. Analysis of two of these inclusions (Table 1) shows a high Cr and Ni (9,400 p.p.m.) content, considerably more than the clay in which the spherules were found. As iridium generally correlates positively (above zero in impact melts) with Ni (refs 9, 10) we expect the inclusions to have a high Ir content. The whole rock INAA analysis of the spherules confirms the high K/Na ratio. Co, Cr, Ni, As and Sb occur in high concentrations and are presumably concentrated in the opaque inclusions. REE values are low. Similar low values in K-feldspar are rarely reported¹¹.

A preliminary K/Ar dating (E. A. Hebeda, personal communication) on handpicked and ultrasonically cleaned, but

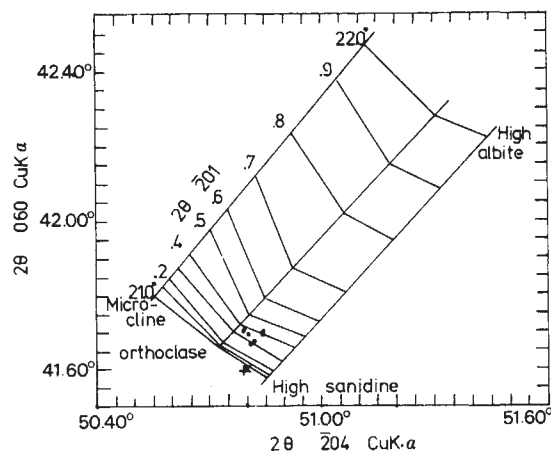


Fig. 1 Plot of 2θ angles (204) and (060) on Wright's¹⁹ structural state diagram of the spherules (+) and reported^{16,17} authigenic sanidine (●). The 2θ angle (201) of the sanidine spherule predicted from this diagram (20.9°) hardly differs from the measured 2θ (201): (20.98°). Authigenic sanidine shows 2θ (201) of 20.95°–21.01°, well outside the predicted range of 21.3°–21.42°. x, Sanidine phenocrysts in Colomera iron-meteorite²³.



Fig. 2 Thin sections of the sanidine spherules from the Cretaceous–Tertiary boundary, showing dendritic microstructure and fibres radiating from the surface and from opaque inclusions. Scale bar, 75 μm .

otherwise untreated sanidine spherules, gave a minimum age of 44 ± 2 Myr. This is ~ 21 Myr less than the generally accepted age of 65 Myr for the K–T boundary¹², and is probably due to loss of argon or to impurities within the spherules.

Figures 2 and 3 show the microstructure and crystal structure of the spherules. The microstructure consists of minuscule dendrites and fan-shaped fibres, radiating from the surface into the spherule, and from the opaque inclusions, acting as nuclei (Fig. 2a–c). This texture suggests that crystallization took place rapidly¹³.

But how did the spherules originate? Authigenic growth of K-feldspar as microcline or adularia occurs commonly^{14,15}, but little information on authigenic sanidine is available^{16–18}. Authigenic growth of the spherules, however, is considered unlikely, because of the crystal shape, texture and structural state of the sanidine.

Authigenic crystallization takes place slowly, which usually leads to equidimensional, euhedral crystals, in strong contrast with the texture shown in Figs 2 and 3. Figure 1 shows the difference in structural state¹⁹ between the sanidine of the spherules and authigenic sanidine—authigenic sanidine is clearly anomalous. The only argument for authigenic growth would be the high K/Na ratio, because of the limited miscibility of K- and Na-feldspar at low temperatures¹⁵.

A volcanic origin for the spherules is also considered unlikely, because of the lack of other volcanic products at the K–T boundary, and the high K/Na ratio.

A relation with the terminal Cretaceous impact event remains the most plausible explanation. We may consider the spherules either as microtektites, which implies a terrestrial source rock, or as ablation products of the incoming projectile(s). Mikrotektites are normally glassy objects with a terrestrial chemical composition, ejected from an impact crater²⁰.

The initiation of crystallization at the surface of the spherules indicates that the spherules were already in their present, spherical shape when crystallization started. This favours strongly rapid cooling, beginning at the surface of a molten silicate droplet. Perhaps the 'monomineralic' composition of the melt of the spherules has led to dendritic crystallization, instead of glass. For microtektites the high K/Na ratio would be difficult to explain, although K enhancements in crater ejecta and impact melts have been observed²¹.

The texture of the spherules resembles the texture of chondritic ablation spherules reported by Blanchard *et al.*²², although the chemistry of present spherules is completely different. Ablation products, rich in K are not yet reported; K is an accessory element in all types of meteorites (150–1,400 p.p.m., ref. 6), and only from a few iron meteorites are K-feldspar phenocrysts reported²³ with the same X-ray pattern as the spherules (Fig. 1). The enriched chalcophile elements As, Se and Sb may be derived from a comet, which is supposed to have preserved (part of) its volatile constituents. K may similarly be

Table 1 X-ray diffraction, microprobe and instrumental neutron activation analysis of sanidine spherules from the base of the Cretaceous–Tertiary boundary clay at the Barranco del Gredero, Caravaca, south-east Spain

Microprobe				Instrumental neutron activation		X-ray diffraction (CuK α)	
	Mean of 5 spherules (%)	Dark inclusions		Sanidine spherules (p.p.m.)	Clay between spherules (p.p.m.)	Lattice constants	
SiO ₂	64.3 $\pm$ 0.2	41.0	36.75	Na	700	313.6	
Al ₂ O ₃	17.7 $\pm$ 0.4	17.8	17.7	K	10.2%	0.95%	
TiO ₂	—	0.1	0.1	Sc	3.49	11.3	
Cr ₂ O ₃	—	0.15	0.15	Cr	269.3	952	α 90.000°
FeO	(0.05–0.7)	15.05	19.55	Fe	1.77%	8.04%	β 116.026°
MnO		0.05	0.05	Co	184.1	720	γ 90.000°
NiO		0.8	1.2	Ni	810	2,980	
MgO		3.2	2.55	Zn	390	1,740	
CaO		1.25	1.2	As	166	751	a 8.61
Na ₂ O	0.15 $\pm$ 0.015	0.35	0.4	Se	<3	6.0	b 13.02
K ₂ O	16.0 $\pm$ 0.2	0.8	0.65	Sb	4.8	17.2	c 7.19
ZnO	—	0.25	0.2	La	1.35	5.5	V 724.058
				Ce	5.7	14.5	
Total	98.45%	80.8%	80.5%	Sm	0.27	0.9	2θ
				Eu	0.22	0.271	$\bar{2}01$ 20.98°
				Hf	1.9	4.8	131 ($\bar{1}\bar{3}1$) 29.80°
				Th	4.8	10.2	060 41.60°
				Ir	<11 p.p.b.	44.0 p.p.b.	$\bar{2}04$ 50.76°

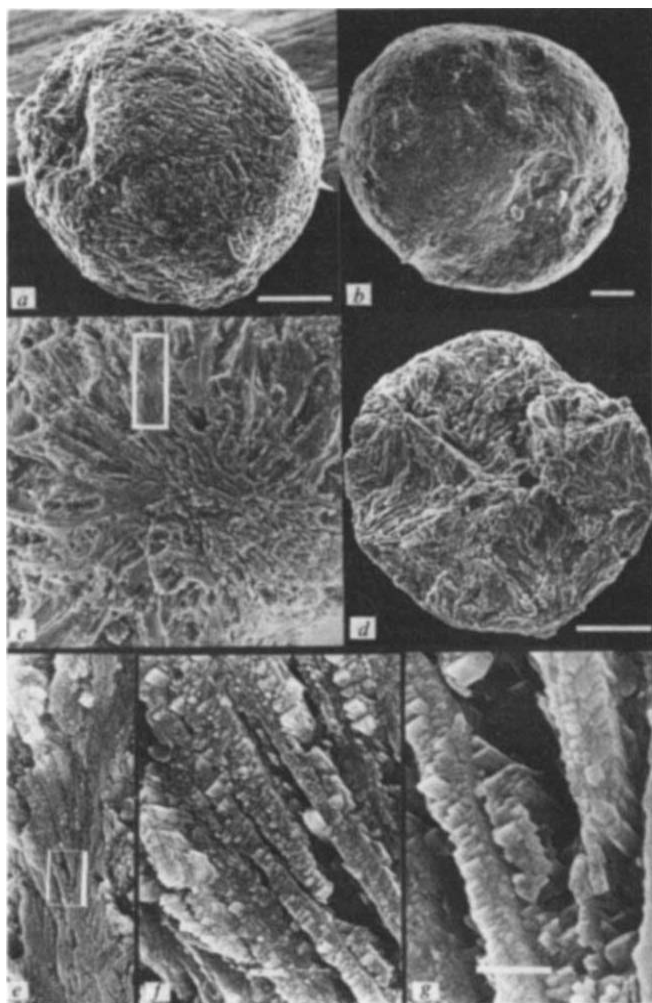


Fig. 3 Scanning electron micrographs showing the morphology of the sanidine spherules and dendritic sanidine crystals. Scale bars: a, b, d, 100 μm ; f, g, 1 μm . e–g are enlargements of c.

derived from a comet. However, K-feldspar is a differentiated mineral, which probably does not occur in undifferentiated Solar System material, as comets are supposed to. Bandhari and Shah's report²⁴ of K-rich globules from Luna-20 soil is notable as the soil of the Moon is supposed to contain meteoritic and cometary material (J. Hertogen, personal communication). A third possibility is that the KAlSi_3O_8 spherules are condensates of more volatile constituents, which are vaporized on entry to the atmosphere or on impact on the Earth.

We suggest that sanidine spherules, restricted to the 'fall-out' lamina of the terminal Cretaceous impact event, are derived from a projectile, rich in chalcophile and moderately volatile elements like K, As, Se, Sb and Zn. Further chemical analysis of the spherules and their inclusions may shed light on the terminal Cretaceous impact event and on the composition of the impacting body.

We thank J. J. Hermes, J. E. van Hinte, E. A. Hebeda, S. Pen, M. de Bruin and many others for their contributions. The ZWO-WACOM Electronmicroprobe Laboratory at the Free University and the Interuniversity Reactor Institute were used for chemical analyses.

Received 12 January; accepted 11 May 1981.

1. Alvarez, L. W. *et al.* *Science* **208**, 367–371 (1980).
2. Smit, J. & Hertogen, J. *Nature* **285**, 198–200 (1980).
3. Ganapathy, R. *Science* **209**, 921–923 (1980).
4. Kyte, F. T. *et al.* *Nature* **288**, 651–656 (1980).
5. Hsu, K. J. *Nature* **285**, 201–203 (1980).
6. Mason, B. *Handbook of Elemental Abundances in Meteorites* (Gordon and Breach, New York, 1973).
7. Smit, J. *Proc. Kon. Ned. Akad. Wet.* **80**, B, 280–301 (1977).

8. Borg, I. Y. & Smith, D. K. *Geol. Soc. Am. Mem.* **122** (1969).
9. Palme, H. *et al.* *Proc. 10th Lunar Sci. Conf.* 2465–2492 (1979).
10. Lambert, P. *Impact and Explosion Cratering*, 449–460 (Pergamon, Oxford, 1977).
11. Smith, J. V. *Feldspar Minerals* (Springer, New York, 1974).
12. Van Hinte, J. E. *Bull. Am. Ass. petrol. Geol.* **60**, 498–516 (1976).
13. Lofgren, G. in *Physics of Magmatic Processes*, 487–551 (Princeton University Press, 1980).
14. Kastner, M. *Am. Miner.* **56**, 7–8, 1403–1442 (1971).
15. Baskin, Y. J. *Geol.* **64**, 132–155 (1956).
16. Woodard, H. H. *J. Geol.* **80**, 323–332 (1972).
17. Brus, Z. & Rieder, M. *Acta univ. carol.* **1**, 37–45 (1975).
18. Lancelot *et al.* *Init. Rep. DSDP Leg 11*, 901–950 (1972).
19. Wright, T. L. *Am. Miner.* **53**, 88–104 (1968).
20. O'Keefe, J. A. (ed.) *Tektites* (University of Chicago Press, 1963).
21. Parfenova, O. V. & Yakovlev, O. I. *Impact and Explosion Cratering*, 843–859 (Pergamon, Oxford, 1977).
22. Blanchard, M. B. *et al.* *Earth planet. Sci. Lett.* **46**, 178–190 (1980).
23. Wassermann, G. J. *et al.* *Science* **161**, 684–687 (1968).
24. Bandhari, N. & Shah, V. G. *Proc. Ind. nat. Sci. Acad. A* **45/3**, 199–200 (1977).

Lamellar-zonal bone with zones and annuli in the pelvis of a sauropod dinosaur

R. E. H. Reid

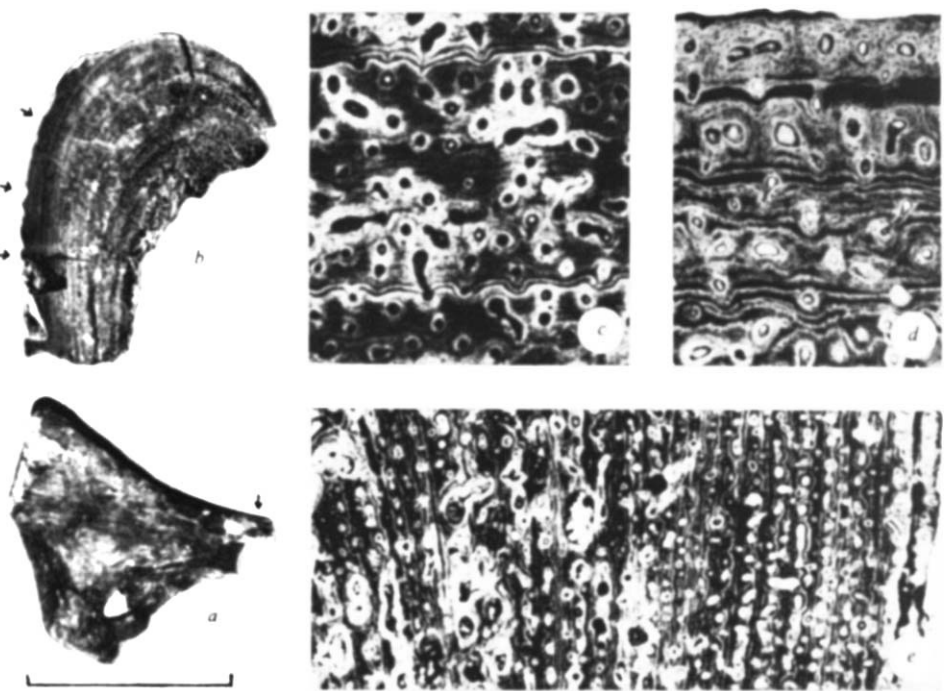
Department of Geology, The Queen's University of Belfast, Belfast BT7 1NN, UK

De Ricqlès^{1–7} distinguishes two principal types of primary (unremodelled) compact bone (compacta), which he terms lamellar-zonal and fibro-lamellar. In the former, bone laid down by accretion at the periosteal (external) surface shows a general coarsely lamellated texture, and vascular canals (for blood vessels) are often sparsely developed and sometimes lacking. Primary osteons, formed by inward growth of bone inside vascular canals, may be present or absent. This type of compacta is seen in bones which grow slowly or only to small sizes, and is the only type seen in most ectotherms. In contrast, fibro-lamellar bone is always rich in primary osteons, set in a matrix of woven bone which is typically laid down rapidly. This type of bone is seen mainly in medium- to large-sized endotherms, and reflects their ability to reach such sizes more quickly than comparable ectotherms. In ectotherms, lamellar-zonal bone may show a cyclical alternation of thin layers of dense bone, termed annuli, with thicker vascular layers known as zones, but it is claimed² that such features are not developed cyclically in endotherms. The presence of fibro-lamellar compacta, without annuli and zones, in various dinosaurs⁷ is hence seen as evidence that dinosaurs were endotherms. However, I report here a case in which compact bone from the pelvis of a dinosaur is instead lamellar-zonal, and shows typical zones and annuli.

The material studied consists of the proximal parts of an incomplete left pubis (Fig. 1a) and ischium (anterior and posterior bones of the lower part of the pelvis) from an undescribed sauropod dinosaur, found in the Northampton Sands Formation (Middle Jurassic, Lower Bajocian) at Harleston, near Northampton, UK. Both bones are broken across their shafts, and the compacta seen in section at the broken ends shows conspicuous concentric annulations (see Fig. 1b), which are due to its formation from a series of circumferential increments. The external surface shows fine longitudinal grooving, and the vascular canals exposed at broken surfaces are predominantly longitudinal. The compacta surrounds a core of spongy bone (spongiosa), containing tubular cavities which again run longitudinally.

In the pubis, the compacta is thickest on the outward-facing surface, and most of the increments are several times thicker on this side than on the inward side. Each increment is underlain by a sharply defined surface, which resembles the external surface. Where these increments are thickest (Fig. 1c), each one typically begins with a thin (for example, 25 μm thick) but well defined layer of bone, in which vascular canals occur mainly over

Fig. 1 Left pubis of a sauropod dinosaur (British Museum (Natural History) exhibit no. R. 9472) from the Northampton Sands Formation near Northampton, UK. *a*, Proximal part of the pubis, inner face, to show the dinosaurian character of the specimen and the source of the sectional material (arrowed), scale 30 cm. *b*, Polished section at the point arrowed in *a*, viewed distally, showing the annulated appearance; arrows mark the positions of traverse lines used in measuring from a parallel thin section (see text and below) $\times 4$. *c*, One increment of the primary compacta and parts of two others, as seen where the increments are thickest (*b*, left-hand side), showing two of the thin basal annuli and one complete vascular zone, $\times 30$. Moving inwards, the outermost 11 such increments have average thicknesses (three measurements) of 0.30, 0.34, 1.06, 1.17, 0.47, 1.04, 2.27, 2.15, 1.14, 1.78 and 1.78 mm, respectively. *d*, The outermost three increments of the compacta as seen where the increments are thinnest (*b*, right-hand side), and part of the underlying fourth, $\times 38$. *e*, Traverse through the compacta where its increments are thinnest, from the external surface (at right) to near the limit of the spongiosa (left), $\times 12$.



grooves in the underlying surface. A second similar layer sometimes follows, usually less distinct. The rest of the increment is formed by strongly vascular bone, showing little or no concentric layering, but packed with primary osteons. The vascular canals of this part of the increment are irregularly interconnected at rather wide intervals, and show no regular arrangement. The increment then ends with a surface which resembles the one underlying it. Up to 13 such increments were counted in a section cut parallel to the surface shown in Fig. 1*b*, with an average thickness (three measurements) of 1.23 mm in the outermost 11 (range 0.30–2.27 mm; see Fig. 1 legend). Where the increments are thinnest (Fig. 1*d,e*), there are usually several thin basal layers, but the vascular parts are reduced, and their vascular networks may be single layered. Bone under the terminal surface may also show a layered appearance, and this extends through the whole depth of some increments. Up to 17 such increments were counted, with an average thickness of 0.35 mm. Intervening parts of the compacta show conditions intermediate between these two types (Fig. 1*c,d*).

A sample from the ischium shows compacta of the type seen in Fig. 1*c*, but only five or six increments remain between the surface and the spongiosa. (As usual, the spongiosa grew outwards by encroaching on the compacta from within, and these different counts of increments reflect different degrees of encroachment.) In both bones, Haversian systems (secondary osteons, formed from bone laid down in vascular canals cut though the primary structure by resorption) are mainly seen near the spongiosa. The bones are iron stained, with their fine structure partly masked as a result. Using cross-polarization, the fine banding of lamellar bone can be found in primary osteons, and in the Haversian bone and spongiosa, but it is not seen in the thin circumferential layers which seem to consist of parallel-fibred bone. Woven bone can be recognized in parts, between primary osteons.

Although these bones are pelvic, their shafts can be compared with those of limb bones, and the concentric structures seen in their compacta reflect the way in which new bone was added at the surface. These structures are not a result of internal remodelling, which is limited to replacement of the primary compacta by Haversian bone and spongiosa. The surfaces between successive increments mark pauses in growth represented by a resting line at the base of each increment as seen in section (see Fig. 1*c*). Each increment, as developed in

Fig. 1*c*, can be interpreted as consisting of a thin basal annulus with a vascular zone outside it; the modified pattern seen in Fig. 1*d* shows the condensing effect of slower growth. These features are repeated cyclically through the whole depth of the primary compacta, up to 17 times down to the level of internal replacement by spongiosa. There is no sign that this growth plan replaced an earlier non-cyclic pattern. Hence, they cannot be dismissed as a result of incidental formation of annuli, or their formation at the end of normal growth (compare ref. 2, p. 109 and ref. 4, p. 53). Annuli are more usually found under resting lines³, but annuli which follow resting lines, as in the material examined here, are known from the pelycosaur *Dimetrodon*, for example (Fig. 4 of ref. 8). The most similar material observed by de Ricqlès (Fig. 3 of pl. 19, ref. 6) is compacta from limb bones of the fossil amphibian *Stenotosaurus* (Subclass Labyrinthodontia, Order Temnospondyli, Suborder Stereospondyli; Lower Triassic), which may show thick vascular zones packed with primary osteons as found in parts of the material examined here (Fig. 1*c*), or a more condensed pattern (Fig. 9 of pl. 5, ref. 2) like that shown here in Fig. 1*d,e*. The compacta from *Stenotosaurus* was specifically shown^{2,6} as being lamellar-zonal bone with zones and annuli; therefore I take this to be the character of the compacta of the bones studied here.

The primary compacta of this sauropod pubis and ischium is thus not of the fibro-lamellar type, thought⁷ to be general in dinosaurs, and shows features (cyclically repeated annuli and zones) described^{2,4,6} as characteristic of ectotherms and absent² from known endotherms and dinosaurs. Their presence here cannot be dismissed as due simply to slow growth of the pelvic bones (compare p. 139, ref. 7), because the presence of resting lines between thick vascular zones implies that periods in which no bone was deposited periosteally occurred alternately with periods of more or less rapid growth. Where the zones are thick (Fig. 1*c*), they are more vascular than is usual in modern reptiles (compare with ref. 9), but can be closely matched in the amphibian *Stenotosaurus*, which is not a likely endotherm (all modern amphibians are ectothermic)—this condition has also been interpreted² as being related to aquatic modes of life. Thus the sauropod discussed here seems to have been ectothermic, unless annuli and zones can be cyclically developed in an endotherm. At first sight, this result is surprising but de Ricqlès' main work^{8,10–13} on bone in fossil amphibians and reptiles contains no systematic study of dinosaurs, and his summary⁷ of their bone

histology makes use of work by other authors. Some material in the theropod *Allosaurus* is thought to show lamellar-zonal example if bone included by Seitz (Figs 53, 54 of ref. 14) from the theropod *Allosaurus* is thought to show lamella-zonal structure. This suggests that the material examined here only seems to be anomalous because no adequate systematic study of dinosaurian bone histology has yet been published.

I thank Drs H. W. Ball and A. J. Charig for permission to study the material, and Dr Charig for identification; Dr A. C. Milner and Mr C. A. Walker for assistance; Professor T. J. Harrison and Dr M. Ferguson for discussion; and The Queen's University of Belfast for travel grants. The sections were cut by Mr W. G. Allingham and Mr S. Watters processed the photographs.

Received 16 January; accepted 1 May 1981.

1. de Ricqlès, A. J. *C. r. hebdomadaire. Séances Acad. Sci., Paris* **275**, 1745–1749 (1972).
2. de Ricqlès, A. J. *Annls Paléont.* **61**(1), 49–149 (1975).
3. de Ricqlès, A. J. *Annls Paléont.* **62**(1), 71–126 (1976).
4. de Ricqlès, A. J. *Annls Paléont.* **63**(1), 33–56, 133–139 (1977).
5. de Ricqlès, A. J. *Annls Paléont.* **63**(2), 133–160 (1977); **64**(1), 85–111 (1978).
6. de Ricqlès, A. J. *Annls Paléont.* **64**(2), 153–184 (1978).
7. de Ricqlès, A. J. in *Morphology and Biology of Reptiles* (eds Bellairs, A. d'A. & Cox, C. B.) 123–150 (London, 1976).
8. de Ricqlès, A. J. *Annls Paléont.* **60**(1), 3–58 (1974).
9. Enlow, D. H. in *Biology of the Reptilia* (eds Gans, C. & Bellairs, A. d'A.) 45–80 (Academic, London, 1969).
10. de Ricqlès, A. J. *Annls Paléont.* **54**(2), 131–146 (1968).
11. de Ricqlès, A. J. *Annls Paléont.* **55**(1), 3–72 (1969).
12. de Ricqlès, A. J. *Annls Paléont.* **58**(1), 17–80 (1972).
13. de Ricqlès, A. J. *Annls Paléont.* **60**(2), 171–234 (1974).
14. Seitz, A. L. *Nova Acta Acad. Caesar. Leop. Carol.* **87**, 230–370 (1907).

New subclass of birds from the Cretaceous of South America

C. A. Walker

Department of Palaeontology, British Museum (Natural History), Cromwell Rd, London SW7 5BD, UK

Current classification of birds recognizes three subclasses which are morphologically distinct: the Archaeornithes for *Archaeopteryx*, the Odontornithes for the Hesperornithiformes and the Ichthyornithiformes, and the Neornithes for all modern birds and their extinct immediate relatives. (Some authorities¹ prefer different names for some of these taxa.) I have examined new material recently discovered in the Upper Cretaceous rocks of Argentina which indicates the existence of a group of birds having features so different from those of the currently recognized subclasses that they seem to represent a fourth subclass, here named the Enantiornithes ('opposite birds'). I describe unique features of the Enantiornithes which include a reduced outer metatarsal, in some forms an extreme modification of the remaining elements of the tarsometatarsus, a highly modified pectoral girdle, and sometimes a characteristic perforation in the proximal end of the humerus.

In the 1970s Dr Jose Bonaparte (Museo Argentino de Ciencias Naturales, Buenos Aires) conducted field excavations in the continental deposits of the Lecho Formation (Salta Group) of El Brete (Salta Province), northwestern Argentina, the age of which is probably Maestrichtian (uppermost Cretaceous). The collection included ~60 individual bones, some associated, which are presumed to be avian; they show no similarity to any of the accompanying dinosaur remains (a titanosaurid, a coelurosaur and a carnosaur)². This bird material consists of all the major postcranial elements and the posterior portion of a left lower jaw ramus; it will be housed in the Universidad Nacional de Tucumán, Argentina. I have described the material here as belonging to members of a single monophyletic group. Although

found separately, most of the elements are of a single unique form: for example, there are five coracoids, all very different from those of other birds, including that of *Archaeopteryx*. The same is true of the 10 humeri, 6 ulnae, 5 femora, 5 tibiotarsi and 5 tarsometatarsi. The hypothesis that all these remains belong to one group is supported by the fact that there is some degree of association between the elements: for example, one articulated group consists of scapula, coracoid and humerus, and in another case a very similar humerus was found with the whole of the rest of the forelimb skeleton. The fore- and hindlimb elements can be associated only tentatively, by size, and by a single associated ulna and tibia (4032).

However, within the highly characteristic structure, both size and shape vary considerably. The material falls into five categories, of which three have about the same dimensions (but differ from each other in form); in the other two categories the material is much smaller and much larger respectively. The morphological differences between the five tarsometatarsi are particularly striking; they clearly represent three lines of evolution so distinct that, had they still been extant, each would probably have been assigned a separate ordinal status. The differences between the humeri are less marked but are nevertheless significant.

The new specimens from Argentina possess certain characters common to all birds. These include a strap-shaped scapula; a coracoidal foramen (=supracoracoidal foramen of Ostrom³); a humerus with proximal and distal expansions almost in the same plane, well developed deltoid and bicipital crests, a pneumatic fossa and/or foramen and a well marked ligamental furrow; a radius with an expanded distal end; an opisthopubic pelvis; a pit in the head of the femur for the attachment of the round ligament; and some degree of fusion of the metatarsals.

A cladistic analysis of the remaining characters of this group, for which the new name Enantiornithes ('opposite birds') is proposed, results in the cladogram shown in Fig. 1. Table 1 lists the synapomorphies common to Enantiornithes and all other post-Jurassic birds (Odontornithes and Neornithes) in which they differ from the corresponding character-states of *Archaeopteryx*, while Table 2 lists the synapomorphies which are shared by the Odontornithes and Neornithes, in which they differ from the corresponding character-states of the Enantiornithes.

The positive character-states unique to the Enantiornithes (nos 3–6a, 8, 10 and 13 in Table 2) make it impossible to place them in any of the three major groups already recognized (Archaeornithes, Odontornithes and Neornithes). There are, however, plesiomorphic similarities with *Archaeopteryx*, including the length and slenderness of the femur and the imperfect fusion of the tarsometatarsus. As I do not support strictly Hennigian classifications I suggest that this group be assigned the status of a subclass, thus fitting it into the traditional classification as coordinate with the other three subclasses.

The coracoid (Fig. 2a, D and E) differs from those of typical birds, not only in having no procoracoid but also in its lack of a prominent sterno-coracoidal process. Perhaps the most fundamental and characteristic difference between the Enantiornithes and all other birds is in the nature of the articulation between the scapula (Fig. 2a, C) and the coracoid, where the 'normal' configuration is completely reversed. The humerus too possesses unique features: for example, the head is almost flat in anconal view (Fig. 2a, B) while there is a well marked depression

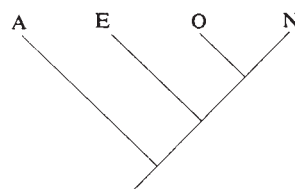


Fig. 1 Suggested cladogram for the class Aves. A, Archaeornithes; E, Enantiornithes; O, Odontornithes; N, Neornithes.

Table 1 List of the synapomorphies shared by the Enantiornithes and all other post-Jurassic birds, in which they differ from the corresponding character-states of *Archaeopteryx*

Character	Archaeornithes	Enantiornithes, Odontornithes and Neornithes
1 Vertebral centra	Not saddle-shaped	Saddle-shaped (? cervicals only in Enantiornithes)
2 Number of sacral vertebrae	Probably 6	At least 9
3 Caudal vertebrae	Not reduced	Reduced to a pygostyle (indication only in Enantiornithes)
4 Sternum	Not developed	Well-developed, with keel
5 Abdominal ribs	Present	Absent
6 Coracoid	Lunate	Elongate, spatulate at sternal end
7 Proximal carpals	Radiale and ulnare of simple shape	Scapholunar and cuneiform of complex shape
8 Distal carpals	Lunate bone only	Trochlea carpalis and extensor process fused together to form part of carpometacarpus, with pollical facet and well-developed metacarpal facet
9 Metacarpals	Unfused II-IV (I-III)	Fused
10 Ilium and ischium	Not fused distally	Usually fused to enclose ilio-ischiadic fenestra
11 Degree of fusion of astragalus and calcaneum to tibia	Nil or only partial (ref. 5)	Complete

Table 2 List of the synapomorphies shared by the Odontornithes and the Neornithes, in which they differ from the corresponding character-states of the Enantiornithes

Character	Enantiornithes	Odontornithes and Neornithes
1 Saddle-shaped vertebral centra	Probably in cervicals only	In most cases, in cervicals and dorsals
2 Procoracoid	Absent	Present
3 Scapular articulation on coracoid	Boss	Facet
4 Coracoid articulation on scapula	Facet	Boss
5 Head of humerus	Flattened profile	Rounded profile, no flattening
6 Palmar surface of humerus:		
<i>a</i> , depression below head	Well marked	Absent
<i>b</i> , brachial depression above distal end	Absent	Well developed, surrounded by scar of brachialis anticus muscle
7 Internal condyle of humerus	Poorly developed	Well developed
8 External condyle of humerus	Transversely orientated	Less so
9 Femur	Relatively long and slender, without fibular groove	Much shorter than tibia, with well-marked fibular groove
10 External cotyla of ulna	More pronounced	Less pronounced
11 Cnemial crests of tibiotarsus	Without flanges	With highly developed flanges
12 Tendinal groove of tibiotarsus	Absent	Present
13 Distal articulation of tibiotarsus:		
<i>a</i> , internal condyle	Bulbous	Less bulbous
<i>b</i> , intercondylar fossa	Narrow	Wide
14 Fusion of tarsometatarsus	Only partial	Complete

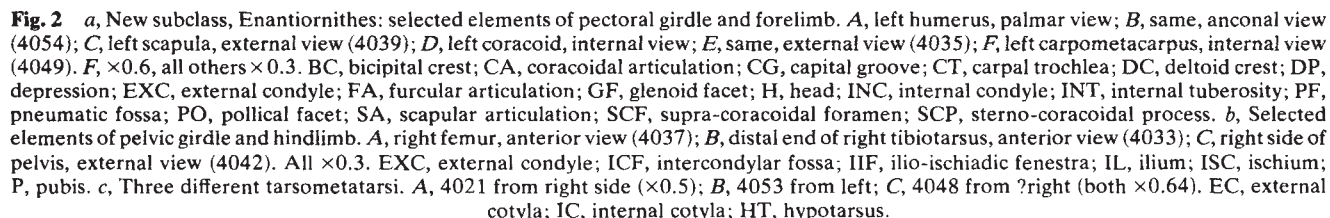
The characters of the new species *Enantiornis leali*, listed in 2-6*a*, are based on an associated coracoid, scapula and humerus (no. 4035). The species has been named after J. C. Leal, who discovered the locality and helped in the collection of material.

distal to it on the palmar side (Fig. 2*a*, A). At the distal end of the humerus the internal and external condyles (articulations for the radius and ulna respectively) are not well formed, the external condyle being oriented more transversely (Fig. 2*a*, A) as in *Alexornis antecedens*⁴ from the Campanian (Upper Cretaceous) of Mexico. Furthermore, there is no indication of either a brachial depression or a scar for the brachialis anticus muscle. The most remarkable feature of the humeri is the variation in the area of the internal tuberosity, where four well preserved specimens show three distinct types. One has the large pneumatic foramen typical of many birds, another seems to have no such structure, while the third has what could be regarded as a pneumatic fossa with no foramen but with a canal running proximo-distally through the internal tuberosity. This third condition seems to be unique as it is unknown in any other bird or reptile. The remaining forelimb elements, although possessing unique features, are typically avian, especially metacarpals II-IV which are fused into a carpometacarpus (Fig. 2*a*, F). Very little can be said about the sternal fragment except that it has a keel and a large sternal notch.

The two preserved pelves are incomplete, but indicate an opisthopubic condition (Fig. 2*b*, C). This configuration,

generally believed to be confined to birds and ornithischian dinosaurs, has now been noted also in certain advanced coelurosaurs of the family Dromaeosauridae which, like the Enantiornithes, are of Maestrichtian age. However, the large ilio-ischiadic fenestra is found only in birds. Furthermore, the smaller specimen has a synsacrum of at least nine vertebrae, a higher number than that found in coelurosaurs, and the last vertebral centrum is so tapered as to give a clear indication of a pygostyle.

The unusual morphology of the hindlimb elements might raise doubts about an avian identification. The femur (Fig. 2*b*, A), for example, could be said to show affinities with that of a very small theropod dinosaur in being long and slender, but in detail it seems to resemble more closely that of *Archaeopteryx*. The tibiotarsus (Fig. 2*b*, B) is very bird-like in having the astragalus and calcaneum fused to its distal end; the same is true of certain coelurosaurs, but the latter, unlike the Enantiornithes and all modern birds, still show marked suture lines between the bones involved. The distal articulation of the tibiotarsus is itself unique in that the internal condyle is larger and bulbous and the intercondylar fossa much narrower than in any modern bird.



It is too early to speculate as to the mode of life of these birds, but the largest forelimb elements indicate an animal with a wingspan of at least 1 m. The flying capabilities were probably limited or even non-existent as the sternum, although keeled, seems to have had a large sternal notch, and the distal articulation of the humerus is only weakly developed. The latter

I thank Dr Bonaparte for allowing me to describe this material, the University of Tucumán for loan of the specimens, and Miss Cassandra Brown for producing the line drawings. The explorations were organized and supported by the Universidad Nacional de Tucumán, the Consejo Nacional de Investigaciones Científicas y Técnicas and the Fundación Miguel Lillo.

1. Brodtkorb, P. *Bull. Fla. St. Mus. biol. Sci.* **7**, 180 (1963).
2. Bonaparte, J. F. & Powell, J. E. *Mém. Soc. géol. Fr.* **139**, 19–28 (1980).
3. Ostrom, J. H. *Smithsonian Contr. Palaeobiol.* **27**, 1–21 (1976).
4. Brodtkorb, P. *Smithsonian Contr. Palaeobiol.* **27**, 67–73 (1976).
5. Tarasitano, S. & Hecht, M. K. *Zool. J. Linn. Soc.* **69**, 149–182 (1980).

Competition relatedness and efficiency

J. R. W. Harris

Natural Environment Research Council,
Institute for Marine Environment Research, Prospect Place,
The Hoe, Plymouth PL1 3DH, UK

Seger¹ has argued that, if a law of diminishing returns of personal fitness² with increasing consumption of a limiting resource applies, a greater increment to inclusive fitness³ may accrue to an individual by sharing the resource with its relatives than by excluding them. That is, from the point of view of an individual's inclusive fitness, there will exist an optimal relation between resource abundance, conversion efficiency (in terms of increment in personal fitness per resource unit consumed) and competitor abundance and relatedness to the subject. Here, this is rendered more concrete by deriving expressions for the optimum consumption rate for any one of a number of related individuals competing for a finite resource.

Let the selective universe consist of N individuals, among which the relatedness⁴ of the j th to the i th is z_{ij} . Let a total consumption x of a particular resource by the i th individual result in a total net reproduction for that individual of $g_i(x)$. Then, if this individual consumes an amount p_i , let the inclusive net reproduction, r_y^* , of the y th individual be defined by

$$r_y^* = \sum_{i=1}^N z_{iy} g_i(p_i) \quad (1)$$

(Note that the use of 'inclusive' here does not correspond exactly to its use in the original definition of 'inclusive fitness' by Hamilton³. It avoids the necessity for a distinction between social and other effects and apparently corresponds to his earlier, heuristic usage⁵.) Differentiating and setting the derivative equal to zero yields a turning point with respect to p_y when

$$g_y(p_y) = - \sum_{i=1, i \neq y}^N x_{iy} g_i(p_i) dp_i/dp_y \quad (2)$$

Let the total amount of resource available to, and divided between, the N individuals be fixed, so that the p_i values are linearly dependent,

$$\sum_{i=1}^N dp_i/dp_y = 0, \quad \text{that is,} \quad \sum_{i=1, i \neq y}^N dp_i/dp_y = -1.$$

If the individuals are ordered so that a number n , less than N , can be defined such that $dp_i/dp_y = 0$ if and only if $i > n$, this can replace N both in these summations and in equation (2) without loss of generality. Now, defining s_{iy} as $z_{iy} g'_i(p_i)$ and $\bar{s}_{iy}$ as

$$\sum_{i=1, i \neq y}^n s_{iy}/(n-1)$$

equation (2) may be expanded to give

$$g'_y(p_y) = \bar{s}_y + \sum_{i=1, i \neq y}^n (\bar{s}_y - s_{iy}) dp_i/dp_y \quad (3)$$

If $g''_i(p_i) < 0$ (that is, $g_i(x)$ is locally a law of diminishing returns) for $0 < i \leq n$, then it is sufficient that $d^2 p_i/dp_y^2$ and s_{iy} are not negatively correlated for this to define a maximum. As long as $N \gg n$, so that $\sum_{i=1}^N g_i(p_i)$ is effectively independent of $g_y(p_y)$, this also defines a maximum in $r_y^*/\sum_{i=1}^N g_i(p_i)$, the proportion of the gene pool of the next generation formed by genes identical by descent to those of the y th individual.

Assumption of a particular form for $g_i(p_i)$ can yield an intuitively clearer relation. Thus, a reasonable and flexible assumption is that total net reproduction is a Michaelis-Menten function of consumption minus some maintenance factor (γ_i)

$$g_i(x) = \alpha_i \beta_i (x - \gamma_i) / (\alpha_i (x - \gamma_i) + \beta_i) : x > \gamma_i \quad (4)$$

$$g_i(x) = 0 : \text{otherwise}$$

so that

$$g'_i(x) = g_i^2(x) / \alpha_i (x - \gamma_i)^2 \quad (5)$$

In these equations, α_i , β_i and γ_i are positive constants. If $E_i(x)$ is defined by

$$E_i(x) = g_i(x) / (x - \gamma_i) \quad (6)$$

it may be conceived, in terms of Wiegert⁶, as a net efficiency of net reproduction per unit of consumption. If it is assumed that $p_i \geq \gamma_i$ for all i , that is, no individuals exist on a quota below maintenance, equation (5) may be substituted in equation (3). If, in addition, α_i , the maximum net efficiency, is constant over all individuals, the result may be expressed in terms of E_i

$$E_y^2(p_y) = \bar{c}_y + \sum_{i=1, i \neq y}^n (\bar{c}_y - c_{iy}) dp_i/dp_y \quad (7)$$

where

$$c_{iy} = z_{iy} E_i^2(p_i) \quad \text{and} \quad \bar{c}_y = \sum_{i=1, i \neq y}^n \frac{c_{iy}}{(n-1)}$$

If dp_i/dp_y and c_{iy} are uncorrelated, the second right-hand term of equation (7) disappears

$$E_y^2(p_y) = \sum_{i=1, i \neq y}^n z_{iy} \frac{E_i^2(p_i)}{(n-1)} \quad (8)$$

In the special case in which, in addition, $z_{iy} = z$ for $i \leq n$ and $i \neq y$

$$E_y^2(p_y) = z \sum_{i=1, i \neq y}^n \frac{E_i^2(p_i)}{(n-1)} \quad (9)$$

That is, if an isolated population of equally related individuals can be defined, and success is to be judged in terms of some infinite, external gene pool, each should strive to raise its consumption until the ratio of its squared net efficiency to the mean square net efficiency of its competing relatives is equal to their relatedness.

In many cases, such a simple view would be inappropriate; correlations between dp_i/dp_y and c_{iy} (or s_{iy}) may be expected to be the rule. A positive correlation implies that the subject's consumption reduces most the consumption of competitors whose contribution to its inclusive fitness is thereby reduced least. As $g_y(x)$ is a law of diminishing returns, this will tend to increase optimum consumption and, under equation (4), to reduce optimum efficiency. Such an effect might be produced by a social system such as a pecking order in which the lowest members (least productive) are most affected by vagaries in consumption by those higher in the order. Hence, other things being equal, such a structure would be expected to be associated with lower population efficiencies. A spatial effect would be a common source of the converse, negative correlation, tending to raise optimum efficiency. For many organisms, consumption will tend to decline away from some 'centre of activity'. Competitor relatedness may be expected to decline similarly⁷; hence, the subject's consumption affects most that of those whose contribution to its inclusive fitness is most sensitive.

The negative relation of $g'_i(x)$ to $g_y(x)$ facilitates qualitative understanding of the implications of equation (3). For example,

it may be recognized that increased levels of relatedness between the individuals of a population will tend to reduce their optimal consumption, increasing their optimal net efficiency (the extent to which any one achieves this optimum depends, of course, on its competitors). Similarly, it may be seen that a reduction in the general level of individual consumption itself reduces the optimum level for any one. This immediately implies that it is adaptive for consumption to decline with increasing density of related competitors. Under equation (10), subsuming equation (5), the decline would be hyperbolic. Declines of this or a similar form have been reported for some predators⁸ and, equating hosts with the resource and oviposition with potential consumption, they are well known for insect parasitoids⁸.

Parasitoids have also been found to emigrate in response to the presence of conspecifics⁸; as well as acting as a constraint on optimal consumption, the necessary relation of competitor numbers to individual consumption which is imposed by an overall resource limit implies that these may themselves be optimized. Thus, in the situation to which equation (9) applies, if it is further assumed that p_i is a constant, p_c , and that $g_i(x)$ is the same for all $i < n$ ($i \neq y$), then, from the point of view of any individual, there is an optimum density of competing relatives when $g'(p_c) = g(p_c)/p_c$. Where $g(p_c)/p_c$ may be interpreted as the gross efficiency of net reproduction per unit of consumption⁸. As $p_c \geq 0$ and $g''(p_c) < 0$ the implication is that this is maximized. Territoriality might be interpreted as the result of selection under some such relation for a population density which would be optimal for all competing individuals, although within which each will compete to increase its resource share in the manner indicated by equation (9).

Although a decline in the net efficiency of growth or reproduction at high consumption rates occurs widely⁹⁻¹⁶, there are exceptions¹⁷. These might indicate species for which, in response to the kind of coincidence of interests just outlined, it is the density of related competitors rather than their individual quotas which tends to vary with resource abundance, and for which there is an (evolutionary) expectation that a superfluity of resource will be utilized by an influx of related individuals, that is, inbreeding species exploiting idiosyncratic resources. In these cases, selection might act to produce an ingestive ceiling sufficiently low to veil the putative decline in efficiency.

It is the essence of these arguments that whereas in heavily outbreeding species selection will tend to maximize personal fitness, in more inbreeding ones personal fitness will tend to be sacrificed for increased 'population fitness'. It has been argued that species so adapted can only succeed if they have a fugitive way of life¹⁸. So, if the demands of inter- and intraspecific selection are to be reconciled, an association between this mode of existence and relative inbreeding would be expected; such an association seems to be found^{19,20}. In view of this, the relations derived here imply in addition that fugitive species will tend to exhibit higher net efficiencies of net reproduction per unit of resource depletion.

A potent cyclic hexapeptide analogue of somatostatin

Daniel F. Veber*, Roger M. Freidinger*, Debra Schwenk Perlow*, William J. Paleveda Jr*, Frederick W. Holly*, Robert G. Strachan*, Ruth F. Nutt*, Byron H. Arison†, Carl Homnick*, William C. Randall*, Monroe S. Glitzer†, Richard Saperstein† & Ralph Hirschmann*†

Merck Sharp and Dohme Research Laboratories, *West Point, Pennsylvania 19486 and †Rahway, New Jersey 07065, USA

Conformational analysis has resulted in the design and synthesis of somatostatin analogues which show increased duration of action¹⁻³. The introduction of covalent conformational constraints and elimination of amino acids that are not required led to the synthesis of the highly active bicyclic analogue I, cyclo(Aha-Cys-Phe-D-Trp-Lys-Thr-Cys)^{2,3}, which showed potency equal to or greater than that of somatostatin for the inhibition of growth hormone release *in vitro* and *in vivo*, as well as for the inhibition of glucagon and insulin release *in vivo* (Aha, 7-aminoheptanoic acid). These results suggested that the amino acids -Phe-D-Trp-Lys-Thr- of this analogue and the corresponding residues 7-10 of somatostatin contain all the elements necessary for the expression of the above biological activities, and that the fragment -Cys-Aha-Cys- serves as a conformational constraint, allowing the tetrapeptide to attain a bioactive conformation. It was concluded that such constraint also results in the observed reduced susceptibility to metabolism by peptidases such as trypsin and has permitted both a long duration of action and oral activity^{2,3}. If the sole purpose of the -Cys-Aha-Cys- sequence is indeed only conformational constraint, then it should be possible to design alternative, simpler constraining moieties. We have used a computer modelling and graphics system⁴ to examine possible alternative molecular fragments and report here the synthesis of a highly active cyclic hexapeptide analogue of somatostatin.

Proton NMR studies have resulted in a precisely defined model for the solution conformation of I (refs 2, 5). Because of the diminished molecular flexibility of this molecule, it has been proposed that this model may also be close to the 'bioactive conformation' (ref. 2). Using these defined coordinates and the Merck computer modelling system, analogue I was divided at the bonds indicated in Fig. 1, eliminating the -Cys-Aha-Cys- portion and leaving the tetrapeptide fragment in the 'bioactive conformation'. Various bridging units were examined as possible choices to tie together the free ends of the tetrapeptide unit. Visualization of this process was aided by the use of a structure superposition program in the Merck Molecular Modelling

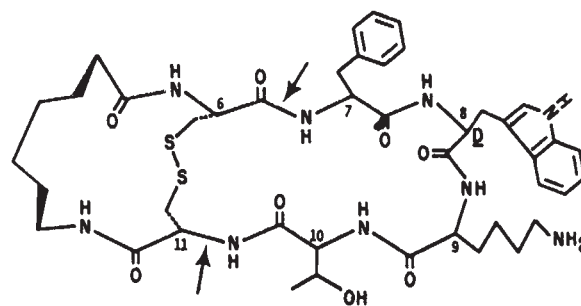


Fig. 1 Cyclo(Aha-Cys-Phe-D-Trp-Lys-Thr-Cys) I, an analogue of somatostatin which shows high biological potency. For computer studies of alternate bridging groups, the molecule was separated at the bonds marked by the arrows.

Received 7 November 1980; accepted 30 April 1981.

- Seger, J. *Nature* **262**, 578-580 (1976).
- Orlivo, M. J. *J. theor. Biol.* **81**, 577-586 (1979).
- Hamilton, W. D. *J. theor. Biol.* **7**, 1-52 (1964).
- Michod, R. E. & Hamilton, W. D. *Nature* **288**, 694-697 (1980).
- Hamilton, W. D. *Am. Nat.* **97**, 354-356 (1963).
- Wiegert, R. G. *Ecol. Monogr.* **34**, 217-241 (1964).
- Meleocot, G. *Les Mathématiques de l'Hérédité* (Masson et Cie, Paris, 1948).
- Hassell, M. P., Lawton, J. H. & Beddington, J. R. *J. Anim. Ecol.* **45**, 135-164 (1976).
- Laybourn, J. E. M. & Stewart, J. M. *J. Zool. Lond.* **174**, 277-283 (1974).
- Armstrong, J. T. *Ecology* **45**, 361-365 (1964).
- Starkweather, P. L., Gilbert, J. J. & Frost, T. M. *Oecologia* **44**, 26-30 (1979).
- Widdows, J. *J. mar. Biol. Ass. U.K.* **58**, 109-124 (1978).
- Checkley, D. M. *Limnol. Oceanogr.* **25**, 430-446 (1980).
- Griffiths, D. J. *Anim. Ecol.* **49**, 99-125 (1980).
- Usher, M. B., Davis, P. R., Harris, J. R. W. & Longstaff, B. C. in *Population Dynamics* (eds Anderson, R. M., Turner, B. D. & Taylor, L. R.) 359-384 (Blackwell, Oxford, 1979).
- Paloheimo, J. E. & Dickie, L. M. *J. Fish. Res. Bd Can.* **23**, 1209-1248 (1966).
- Lawton, J. H., Hassell, M. P. & Beddington, J. R. *Nature* **255**, 60-62 (1975).
- Maynard Smith, J. *Nature* **201**, 1145-1147 (1964).
- Levin, D. A. *Am. Nat.* **109**, 437-451 (1975).
- Gelesener, R. R. & Tilman, D. *Am. Nat.* **112**, 659-673 (1978).

Table 1 Characterization of somatostatin analogues (cyclo(A-B-Phe-D-Trp-Lys-Thr))

	Compound		Amino acid analysis							Purity HPLC	δ Lys γ -CH ₂	Inhibition of growth hormone release (relative potency)
	A	B	A	B	Phe	Trp	Lys	Thr	$[\alpha]_D^{22}$			
1	Ala	Pro	1.00	1.02	1.03	0.98	1.04	0.92	-107.0	98	0.39, 0.55	0.06 (0.03, 0.12)
2	Pro	Pro	—	2.02	0.99	0.95	0.99	0.99	-171.6	97	0.33, 0.53	0.008 (0.003, 0.02)
3	D-Ala	D-Pro	0.99	0.99	0.99	—	1.00	1.02	—	100	0.2–0.7*	0.006 (0.003, 0.01)
4	D-Ala	Pro	0.99	1.05	1.00	0.98†	0.97	0.99	-61.8	93	0.30, 0.45	<0.002
5	Pro	Ala	1.02	1.00	0.98	0.92†	1.00	1.02	-83.9	97	0.23, 0.45	<0.002
6	Pro	D-Ala	1.03	1.02	1.00	1.06†	1.03	0.96	-12.6	96	0.43, 0.60	<0.002
7	Aib	Pro	1.04	1.01	0.99	0.85†	1.00	0.98	-25.5	88	0.20, 0.43	<0.002
8	Phe	Pro	—	0.97	2.04	0.97	0.99	1.03	-23.6	98	0.40, 0.55	1.74 (1.31, 2.32)
9	Phe	Phe	—	—	2.98	—	0.99	1.04	-56.9	93	0.23, 0.44	0.27 (0.22, 0.33)
10	Phe	D-Phe	—	—	3.04	0.93	0.99	1.03	-21.5	95	0.59, 0.68	0.22 (0.20, 0.25)
11	D-Phe	Pro	—	1.00	1.99	0.85	1.00	0.99	-80.1‡	96	0.30, 0.44	<0.002
12	Pro	D-Phe	0.97	—	2.01	0.84†	0.99	1.02	-27.4	99	0.45, 0.61	0.03 (0.03, 0.03)
13	D-Phe	D-Pro	—	1.05	1.96	0.87	0.98	1.01	-17.7	93	0.56, 0.71	<0.002
I	Cys Aha Cys	—	—	—	—	—	—	—	—	—	0.32, 0.48	1.24 (0.81, 1.88) ³

For optical rotation, $c = 1$ in methanol. Reverse phase chromatography was used to estimate purity from the area under the curve of the UV trace at either 210 or 280 nm. Amino acids were analysed after hydrolysis for 20 h in 6 M HCl at 110 °C. Inhibition of spontaneous growth hormone release was evaluated by incubation of isolated pituitary cells with somatostatin or analogue at graded doses (at least 6 doses per analogue) ranging from 10^{-10} – 10^{-5} M. Three replicate plates were used at each dose level. After 4 h incubation, growth hormone levels released into the medium were determined by a double antibody radioimmunoassay for rat growth hormone. Potency of the analogues relative to somatostatin (=1) were calculated using a relative potency formula for parallel line bioassays¹². 95% confidence limits are given in parentheses. This bioassay is essentially as has been described elsewhere¹³. The ED₅₀ for somatostatin in this test is 5×10^{-8} M.

* Two conformers.

† By UV.

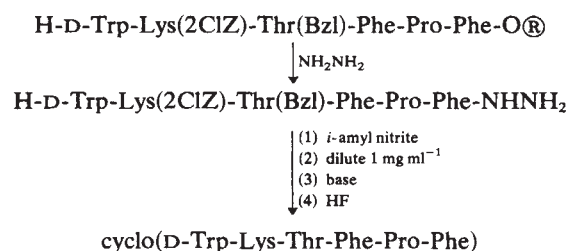
‡ $c = 2$.

System called 'COMPARE' (ref. 4; Fig. 2). Among the molecular fragments examined for completion of the ring were acetyl dipeptide methylamides in the conformations defined for the various types of β -turn⁶. The resulting structure is a cyclic hexapeptide having two β -turns, a common structural occurrence as indicated by X-ray studies of cyclic hexapeptides^{7,8}. A least-squares fit of the terminal overlapping atoms (two atoms at one end, three at the other) was carried out without allowing any bond rotations. Fits ranging in average deviation from 0.38 to 0.61 Å were obtained for the six β -turns of types I–III and I'–III'. Better matches were attained (deviation as low as 0.10–0.31 Å) by allowing some rotation about the bond angles

indicated in Fig. 2, two of which were not defined in the study of the solution conformation of I (ref. 2).

A reasonable match in some cases led us to prepare a number of cyclic hexapeptides having the general structure cyclo(A-B-Phe-D-Trp-Lys-Thr) where A and B were chosen to fit a variety of β -turns either because they have been observed to be part of a β -turn in some X-ray study or because we thought they would be compatible with one or more of the proposed turn types. Alanine, rather than amino acids with more complex side chains, was used in combination with proline in our early analogues, on the assumption that the sole purpose of the A-B unit is conformational constraint.

The cyclic hexapeptides were prepared as outlined below:



The linear peptides were prepared by the solid phase method on a Beckman 990 peptide synthesizer using a protocol described elsewhere⁹. The cyclization procedure also follows basically the procedure described for other cyclic peptide analogues of somatostatin¹. The analogues were characterized as shown in Table 1. In addition to a consistent amino acid analysis, each compound showed an appropriate NMR spectrum. The position of the γ -CH₂ of lysine is given for each compound (Table 1) as the unusual upfield shift is indicative of a proximity of the lysine and tryptophan side chains, a feature which has been postulated as important for biological activity⁵. Proximity of the two side chains was achieved in all the cyclic hexapeptides which suggests a similar conformation in this region of the various peptides.

Each analogue was evaluated for inhibition of the release of insulin, glucagon and growth hormone¹. The results for inhibition of growth hormone release *in vitro* are given in Table 1. (For most of the low-activity analogues, all other bioassays showed no activity at the doses tested.) Table 1 shows that cyclic

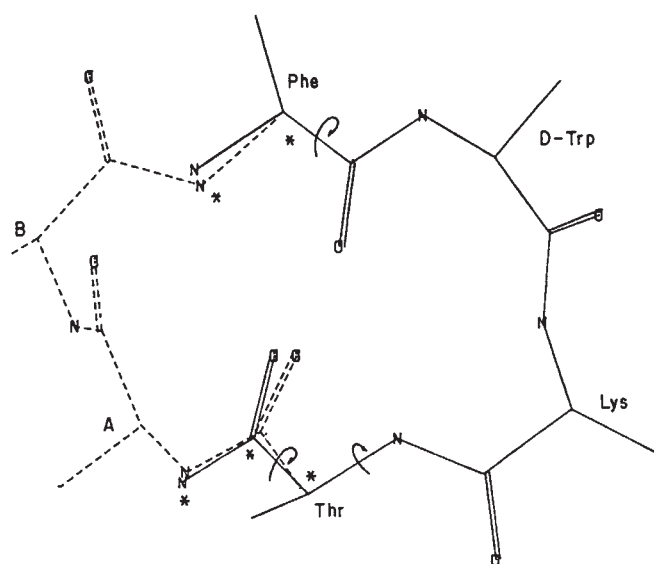


Fig. 2 Computer matching of the termini of a tetrapeptide unit and an acetyl dipeptide-N-methylamide unit having the conformation of a type 3 β -turn. The tetrapeptide unit has the conformation suggested for the -Phe-D-Trp-Lys-Thr- portion of the proposed bioactive conformation of somatostatin analogues². A least-squares fit of the atoms marked by an asterisk was carried out while simultaneously allowing rotation about the bonds marked by arrows. The average deviation of the matched atoms in this case was 0.20 Å.

hexapeptides 1–3 have real activity but only low potency. However, even the most active analogue of this group (1) shows only 6% the potency of somatostatin. We considered that this low potency was due to elimination of an important contributor to receptor binding rather than a failure to attain the correct conformation for the bioactive tetrapeptide fragment. The most reasonable possibility was that we had eliminated a hydrophobic binding element. We therefore prepared several analogues incorporating phenylalanine rather than alanine in the A–B dipeptide unit (compounds 8–13 of Table 1) including an analogue (9) which constitutes cyclization of the sequence 6–11 of [D-Trp⁸] somatostatin. Most striking is the inhibition of growth hormone release *in vitro* by analogue 8 compared with that of analogue 1 (Table 1); the former is almost twice as potent as somatostatin and 29 times as potent as 1 on a molar basis. This *in vitro* test comes closest to measuring binding affinity as enzymatic inactivation is likely to be minimized. The fact that circular dichroism spectra of 1 and 8 in the region 190–250 (Fig. 3) are very similar indicates that these two molecules have a similar peptide backbone conformation and suggests that the higher activity of 8 results from interaction of the additional benzene ring with receptors.

The PMR spectrum of 8 shows features indicative of a high degree of molecular rigidity in the backbone and side chains. A solution conformation has been derived which will be reported elsewhere. The molecular rigidity seems to have lowered the susceptibility of this molecule to the action of trypsin, as was observed for I. Analogue 8 is cleaved by trypsin only slowly, at about the same rate as that of I in identical conditions³. The special importance of proline to the high potency of the cyclic peptide 8 is seen by comparison with 9. The increased potency of 8 seems to be due to the presence of the *N*-substituted cyclic amino acid not present in 9, probably through an influence on the conformation of the cyclic hexapeptide. The altered circular dichroism of 9 in the region of amide bond absorbance (200–250 nm) supports this conclusion (see Fig. 3).

The *in vivo* potency (by weight) of 8, relative to somatostatin, for inhibition of growth hormone release in rats stimulated by pentobarbital is ~20–25 (see Table 2 legend for method). (Variability in this test precludes evaluation of 95% confidence limits.) Potency (by weight), relative to somatostatin, for inhibition of insulin release is 5.2 (2.4, 11), and for inhibition of glucagon release is 8.0 (1.4, 60.2) as measured in the urethane-anesthetized rat. (Inhibition of insulin and glucagon release was measured, relative to somatostatin (=1.0), by the ability to decrease the blood levels of glucagon and insulin in urethane-anesthetized rats as described elsewhere¹. Saline or peptide

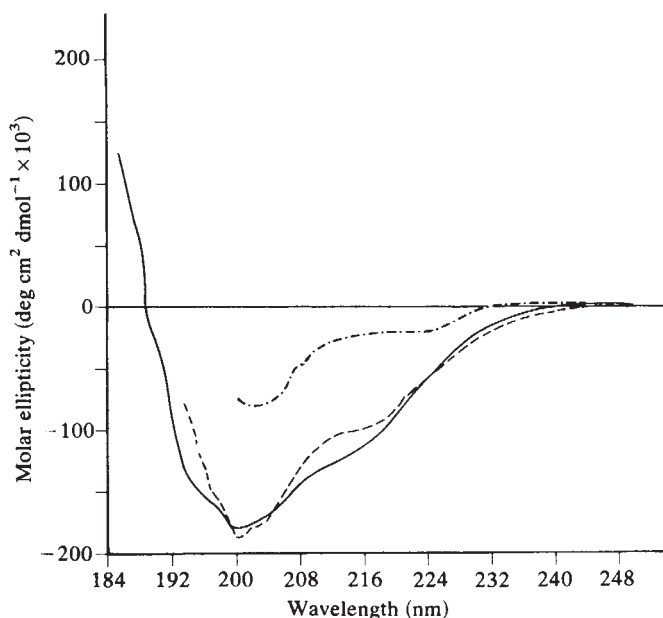


Fig. 3 Circular dichroism spectra of cyclo(Phe-Pro-Phe-D-Trp-Lys-Thr) 8 (—); cyclo(Ala-Pro-Phe-D-Trp-Lys-Thr) 1 (---); and cyclo(Phe-Phe-Phe-D-Trp-Lys-Thr)⁹ (- · - · -). All spectra taken in phosphate buffer pH 7.4.

were given by the external jugular vein and blood samples were taken 5 min later via the portal vein. Insulin and glucagon levels in the plasma were measured by radioimmunoassay.)

The duration of action of 8 is much longer than that of somatostatin and is comparable with that of the bicyclic peptide I, as shown by the inhibition of stimulated growth hormone release 5 h after subcutaneous (s.c.) injection (Table 2). We have shown that somatostatin does not reduce growth hormone levels 75 min after a 250 µg per kg s.c. dose in this same protocol, whereas I causes significant reductions 135 min after injection of 125 µg per kg (ref. 3). In our study, 8 has a duration comparable with that of I when evaluated simultaneously (Table 2). Both I (500 µg per kg) and 8 (750 µg per kg) show a statistically significant reduction in growth hormone levels 5 h after s.c. administration; 8 is also effective in inhibiting the release of growth hormone 1 and 3 h after oral administration (25 mg per kg) in this same test system (Table 2). There was no suppression

Table 2 Inhibition of growth hormone release by somatostatin analogues *in vivo*

Compound	Dose (µg per kg)	Route of administration	Growth hormone (ng ml ⁻¹)	Time (h)
None	—	s.c.	353 ± 130	5
I Cyclo(Aha-Cys-Phe-D-Trp-Lys-Thr-Cys)	125	s.c.	534 ± 300	5
I Cyclo(Aha-Cys-Phe-D-Trp-Lys-Thr-Cys)	250	s.c.	221 ± 123	5
I Cyclo(Aha-Cys-Phe-D-Trp-Lys-Thr-Cys)	500	s.c.	31 ± 11*	5
8 Cyclo(Pro-Phe-D-Trp-Lys-Thr-Phe)	250	s.c.	207 ± 49	5
8 Cyclo(Pro-Phe-D-Trp-Lys-Thr-Phe)	500	s.c.	470 ± 222	5
8 Cyclo(Pro-Phe-D-Trp-Lys-Thr-Phe)	750	s.c.	9 ± 1*	5
None	—	p.o.	1,064 ± 177	3
8 Cyclo(Pro-Phe-D-Trp-Lys-Thr-Phe)	25,000	p.o.	99 ± 55†	3
None	—	p.o.	1,124 ± 341	1
8 Cyclo(Pro-Phe-D-Trp-Lys-Thr-Phe)	25,000	p.o.	9 ± 4‡	1
None	—	p.o.	1,128 ± 338	1
I Cyclo(Aha-Cys-Phe-D-Trp-Lys-Thr-Cys)	25,000	p.o.	140 ± 48§	1

Inhibition of pentobarbital-stimulated growth hormone release was measured essentially according to a procedure described elsewhere¹. Male Sprague-Dawley rats (190–200 g) were given saline or compound either subcutaneously (s.c.) or orally (p.o.). After the designated time period, sodium pentobarbital was given i.v. and 15 min later the rats were bled from the orbital sinus and the plasma assayed for growth hormone content by radioimmunoassay. The data are expressed as the mean ± s.e.m. (6 rats per group). The control is given for each experimental run.

* $P < 0.05$; † $P < 0.001$; ‡ $P < 0.01$; § $P < 0.02$.

when somatostatin was given orally at this dose, even after a shorter time interval (1 h), whereas the bicyclic analogue I showed statistically significant suppression of growth hormone release 1 h after oral administration at this dose, but not after 2 h.

Thus, a highly active and long-acting cyclic hexapeptide analogue of somatostatin has been designed by replacing 9 of the 14 amino acids of somatostatin with a single proline. The long duration of action of this analogue could render this compound class valuable in tests of the potential application of somatostatin-like compounds in the treatment of juvenile diabetes^{10,11}.

We thank Drs Peter Gund and Graham Smith for help with computer studies and Susan Bergstrand for technical assistance.

Received 2 December 1980; accepted 30 March 1981.

1. Veber, D. F. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 2636–2640 (1978).
2. Veber, D. F. in *Peptides: Proc. 6th Am. Peptide Symp.* (eds Gross, E. & Meienhofer, J.) 409–419 (Pierce Chemical Co., Rockford, Illinois, 1979).
3. Veber, D. F. *et al. Nature* **280**, 512–514 (1979).
4. Gund, P., Andose, J. D., Rhodes, J. B. & Smith, G. M. *Science* **280**, 1425–1431 (1980).
5. Arison, B. H., Hirschmann, R. & Veber, D. F. *Bioorg. Chem.* **7**, 447–451 (1978).
6. Chandrasekaran, R., Lakshminarayanan, A. V., Pandya, U. V. & Ramachandran, G. N. *Biochim. biophys. Acta* **303**, 14–27 (1973).
7. Brown, J. N. & Teller, R. G. *J. Am. chem. Soc.* **98**, 7565–7569 (1976).
8. Hossain, M. B. & van der Helm, D. J. *Am. chem. Soc.* **100**, 5191–5198 (1978).
9. Strachan, R. G. *et al. J. med. Chem.* **22**, 586–588 (1979).
10. Gerich, J. E. *Metabolism* **27**, 1283 (1978).
11. Veber, D. F. & Saperstein, R. A. *Rep. med. Chem.* **14**, 209 (1979).
12. Finney, D. J. *Statistical Methods in Biological Assay* Ch. 4, 99–138 (Griffin, London, 1964).
13. Vale, W. & Grant, G. *Meth. Enzym.* **37**, 5–93 (1980).

Oestradiol, sexual receptivity and cytosol progesterin receptors in rat hypothalamus

Bruce Parsons, Thomas C. Rainbow, Donald W. Pfaff & Bruce S. McEwen

The Rockefeller University, New York, New York 10021, USA

Oestradiol and progesterone regulate feminine reproductive behaviour in rodents. Their administration induces changes of biosynthetic activity in the hypothalamus¹, one of which—the induction of progesterin receptors—is correlated with the induction of sexual receptivity^{2–4}, conveniently indicated (in the rat) by the lordosis reflex of the mounted female. We have attempted to define what might be considered as a minimal regime of oestradiol administration—one which is just ‘sufficient’ for activating the lordosis reflex—and have used this paradigm to measure associated changes in nuclear oestrogen (NOER) and cytosol progesterin (CPRs) receptors in the medio-basal hypothalamus–preoptic area (MBH-POA) of the rat brain. We find that two 1-h exposures to oestradiol are sufficient to induce the lordosis reflex and to increase significantly CPRs in the MBH-POA. An essentially discontinuous pattern of NOERs in MBH-POA can promote these changes.

Female rats ovariectomized for 5–7 days were fitted with 5-mm silastic capsules containing oestradiol, which produced physiological levels of the hormone in serum⁴. For behaviour, each female was assigned to a separate mating group of 5–10 animals. Females received 10 mounts by male rats 24 h after initiation of oestradiol treatment. Four h before testing, each female received 500 µg P (subcutaneously (s.c.) in oil). The lordosis quotient (LQ) was calculated as: number lordoses/number of mounts × 100. Results are means ± s.e.m.

In some behavioural experiments, animals received s.c. injections of the protein synthesis inhibitor anisomycin, dissolved 20 mg ml⁻¹ in saline⁵. For all biochemical studies, four to seven samples were used for each observation, each sample consisting of MBH-POA regions from two animals. The amount of oestradiol bound to nuclear receptors in the MBH-POA was determined by using an *in vitro* exchange assay⁶. NOERs were measured 0.5, 1 and 6 h after implantation of oestradiol capsules, as well as 3 and 6 h after their removal. Results are means ± s.e.m. CPR levels in the MBH-POA were quantified using ³H-R 5020 as the radioligand for an *in vitro* assay⁷. CPRs were measured 24 h after insertion of oestradiol capsules, in the absence of exogenous progesterone. CPRs were measured in animals not exposed to oestradiol (controls); this binding represents a class of CPRs which is not influenced by oestradiol administration or withdrawal⁸. CPRs were also determined in animals which had received s.c. injections of oestradiol benzoate (15 µg daily for 3 days) to induce CPRs to maximal levels. The % maximal induction of CPRs was calculated⁴ as: (³H-R 5020 binding in experimental group) minus (³H-R 5020 binding in control group)/(³H-R 5020 binding in oestradiol benzoate) minus (³H-R 5020 binding in control group).

The effects of oestradiol treatment on sexual receptivity as measured by the LQ score indicate that a discontinuous exposure to oestradiol, in two 1-h segments, is sufficient to activate the lordosis reflex. The LQ score of animals which receive oestradiol continuously for 24 h is 63 ± 12 (Fig. 1*b*) and that of animals which receive oestradiol continuously for 6 h is equivalent at 24 h (Fig. 1*c*). However, animals which receive oestradiol continuously for 3 h are not sexually receptive at 24 h (Fig. 1*d*). Figure 1 (and unpublished data) demonstrates that receptivity comparable with 24 or 6 h of continuous oestradiol treatment is seen if animals receive only two 1-h segments of oestradiol, provided that the second treatment is not less than 4 nor more than 13 h after the end of the first period (Fig. 1*e, f, h*) ('sufficient treatment'). No receptivity is seen in animals which receive two 0.5-h segments of oestradiol (Fig. 1*g, i*) ('insufficient treatment').

We studied oestrogen receptor translocation following 'sufficient treatment' and found that retention of oestradiol by MBH-POA cells is transient even though this oestradiol treatment promotes delayed changes in sexual receptivity. These results may be summarized as follows: (1) The level of NOERs in MBH-POA after 1 h of oestradiol treatment resembles that seen after 6 h continuous treatment (1 h: 228 ± 9 fmol ³H-oestradiol per mg DNA; 6 h: 197 ± 23 fmol ³H-oestradiol per mg DNA), and represents ~60% of the nuclear oestradiol receptor capacity⁹. (2) A second 1-h period of oestradiol treatment, beginning 7 h after the end of the first, produces NOER levels in the MBH-POA comparable with those seen after the first 1-h oestradiol period (data not shown). (3) One hour of oestradiol treatment produces at least twice the level of NOERs as does 0.5 h of oestradiol treatment (0.5 h: 93 ± 11 fmol ³H-oestradiol per mg DNA). (4) Six hours after removal of Silastic capsules, NOER levels in the 1- and 0.5-h treatment groups decrease to ~10% and 7% of receptor capacity, respectively. Thus, an essentially discontinuous pattern of NOERs in MBH-POA is sufficient to activate the lordosis reflex at 24 h. Sufficient to activate lordosis was that NOERs in MBH-POA be elevated to 60% of their capacity, at both an early and late period, as outlined above. Six hours of continuous oestradiol treatment can be regarded as sufficient to activate lordosis because this paradigm contains two such 1-h periods.

Two classes of progesterin receptors have been identified in the rat brain—one which is unaffected by oestradiol treatment, the other induced by oestradiol (ref. 8). Although the physiological significance of these two classes of progesterin receptors is unclear, note that oestradiol induces progesterin receptor synthesis in those areas of the brain known to mediate feminine reproductive behaviour, the MBH and POA. Moreover, a 'threshold level' (25–35% maximal induction) of oestrogen-inducible CPRs in the MBH-POA has been shown to be correlated with

the appearance and disappearance of sexual receptivity⁴. We used several temporal paradigms of oestradiol treatment to study inducible CPRs in the MBH-POA, and found that sufficient treatment significantly elevates inducible CPRs to threshold levels in these brain regions. In animals which receive oestradiol continuously for 24 h, CPRs in MBH-POA increase from an unstimulated level of 8.1 ± 0.3 to an induced level of 12.7 ± 0.7 fmol ³H-R 5020 per mg protein. This increase of approximately 4.6 fmol ³H-R 5020 per mg protein represents a 34% maximal elevation in oestrogen-inducible CPRs, an increase to threshold levels (Fig. 1b). In animals which receive oestradiol continuously for 6 h, CPR levels in MBH-POA increase from an unstimulated level of 8.1 ± 0.3 to an induced level of 11.8 ± 1.2 fmol ³H-R 5020 per mg protein. This increase of ~ 3.3 fmol ³H-R 5020 per mg protein represents a 28% maximal elevation in oestradiol-inducible CPRs, also an

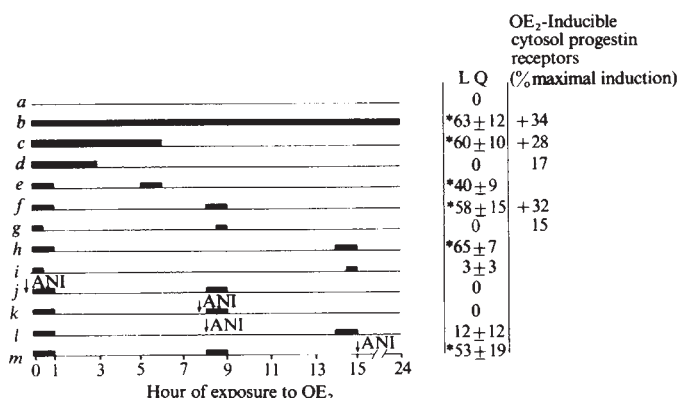


Fig. 1 The effects of oestradiol (OE₂) and anisomycin (ANI) on the lordosis quotient (LQ) and on the induction of cytosol progesterin receptors (CPRs) in the mediobasal hypothalamus-preoptic area (MBH-POA) of the female rat. Biochemical results are expressed as % maximal induction (see text). For behaviour, animals were tested with an experienced male 24 h after the initiation of oestradiol treatment. Four hours before testing, each animal received 500 µg progesterone (s.c. in oil). In some behavioural experiments, animals received s.c. injections of the protein synthesis inhibitor, anisomycin, dissolved 20 mg ml⁻¹ in saline. For CPR measurements, animals were killed 24 h after the insertion of Silastic capsules. No exogenous progesterone was given before death. The temporal paradigms for these experiments are described below. a, No OE₂, control group; b, OE₂ 0–24 h; c, OE₂ 0–6 h; d, OE₂ 0–3 h; e, OE₂ 0–1 h and 5–6 h; f, OE₂ 0–1 h and 8–9 h; g, OE₂ 0–0.5 h and 8.5–9 h; h, OE₂ 0–1 h and 14–15 h; i, OE₂ 0–0.5 h and 14.5–15 h; j, OE₂ 0–1 h and 8–9 h, ANI at –0.25 h; k, OE₂ 0–1 h and 8–9 h, ANI at 8.75 h; l, OE₂ 0–1 h and 14–15 h, ANI at 8 h; m, OE₂ 0–1 h and 8–9 h, ANI at 15 h. We conclude that a discontinuous exposure to estradiol, in two 1-h segments, is sufficient to activate the lordosis reflex and to increase CPRs in the female rat. ANI experiments demonstrate that protein synthesis essential for the activation of the lordosis reflex occurs during the period bounded by and including these two 1-h segments of OE₂ treatment. CPR levels are (fmol ³H-R 5020 per mg protein): a, 8.1 ± 0.3 ; b, 12.7 ± 0.7 ; c, 11.8 ± 1.2 ; d, 10.3 ± 0.7 ; f, 13.3 ± 1.1 ; g, 10.0 ± 0.6 . CPR levels in animals which receive oestradiol benzoate for 3 days before death (15 µg per day) are 21.1 ± 1.2 fmol ³H-R 5020 per mg protein.

increase to threshold levels (Fig. 1c). However, animals which receive oestradiol continuously for 3 h do not show threshold levels of oestradiol-inducible CPRs at 24 h (Fig. 1d); CPR levels in MBH-POA are 10.3 ± 0.7 fmol ³H-R 5020 per mg protein. CPR elevation comparable with 24 or 6 h of continuous oestradiol treatment is seen in animals which receive only two 1-h segments of oestradiol, as outlined above (sufficient treatment). CPRs in this group increase from an unstimulated level of 8.1 ± 0.3 fmol ³H-R 5020 per mg protein to an induced level of 12.3 ± 1.1 fmol ³H-R 5020 per mg protein. This increase of ~ 4.2 fmol ³H-R 5020 per mg protein represents a 32% maximal elevation in oestradiol-inducible CPRs, an increase to threshold levels (Fig. 1f). Threshold levels of oestrogen-inducible CPRs are not seen in animals which receive two 0.5-h segments of oestradiol (Fig. 1g); CPRs in these animals are 10.0 ± 0.6 fmol ³H-R 5020 per mg protein at 24 h. We conclude that a discontinuous exposure to oestradiol, in two 1-h segments, is the minimum period of oestradiol treatment used which is sufficient to elevate inducible CPRs to threshold levels in the MBH-POA of the female rat. Recent work using two widely spaced (14 h) intravenous injections of oestradiol to elevate CPRs supports the notion that a discontinuous oestradiol signal can trigger CPR formation in brain and pituitary¹⁰. These data are consistent with, but not proof of, the concept that one of the neurochemical events mediated by oestradiol which accompanies the activation of behavioural receptivity is the induction of CPRs in the MBH-POA.

We also used anisomycin to demonstrate the time course of the oestradiol-induced changes in protein synthesis which are essential for the activation of the lordosis reflex. It has been shown previously that anisomycin (100 mg per kg) substantially inhibits protein synthesis in the MBH and POA of the female rat for 4 h after administration⁵. If anisomycin (100 mg per kg) is given 15 min before the first (Fig. 1j), 15 min before the second (Fig. 1k), or between the two 1-h segments of oestradiol treatment (Fig. 1l), almost no receptivity is seen at 24 h. However, if the inhibitor is given 6 h after the second period of oestradiol, no significant decrease in LQ is observed at 24 h (Fig. 1m). These data demonstrate that at least a large portion of the protein synthesis essential for the activation of the lordosis reflex is completed within 6 h after termination of the second segment of oestradiol.

The kinetics of oestradiol stimulation of uterine development have been studied by other investigators and offer parallels to our present findings. Anderson and co-workers¹¹ found that oestradiol is retained by uterine nuclei for many hours, whereas oestriol is cleared rapidly. Harris and Gorski¹² showed that although multiple injections of oestriol were as effective in stimulating DNA synthesis at 24 h as a single injection of oestradiol, not all the oestriol injections were required. They suggested that the requirement for oestrogen was not continuous throughout the first 12–15 h, but only at critical phases in the response cycle. Our results suggest that there may also be a discontinuous requirement in brain for oestradiol to activate the lordosis reflex. The appearance of sexual receptivity in the female rat may be determined not only by an initial period of oestrogen-stimulated protein synthesis, but also by a later phase which may be initiated 5–14 h after the prerequisite initial oestradiol exposure.

Received 21 November 1980; accepted 14 April 1981.

- McEwen, B. S., Davis, P. G., Parsons, B. & Pfaff, D. W. *A. Rev. Neurosci.* **2**, 65–112 (1979).
- Blaustein, J. D. & Feder, H. H. *Brain Res.* **177**, 489–498 (1979).
- Mogilevsky, M. & Raynaud, J.-P. *Endocrinology* **105**, 516–522 (1979).
- Parsons, B., MacLusky, N. J., Krey, L. C., Pfaff, D. W. & McEwen, B. S. *Endocrinology* **107**, 774–779 (1980).
- Rainbow, T. C., Davis, P. G. & McEwen, B. S. *Brain Res.* **194**, 548–555 (1980).
- Roy, E. J. & McEwen, B. S. *Steroids* **30**, 657–669 (1977).
- MacLusky, N. J. & McEwen, B. S. *Endocrinology* **106**, 192–202 (1980).
- MacLusky, N. J. & McEwen, B. S. *Nature* **274**, 276–278 (1978).
- McGinnis, M. Y., Krey, L. C., MacLusky, N. J. & McEwen, B. S. *Neuroendocrinology* (in the press).
- Clark, C. R., MacLusky, N. J. & Naftolin, F. *Endocr. Soc. (abstr.)* 129 (1980).
- Anderson, J. N., Peck, E. J. & Clark, J. H. *Endocrinology* **98**, 676–684 (1975).
- Harris, J. & Gorski, J. *Endocrinology* **103**, 240–245 (1978).

* LQ scores in groups a–m were compared with each other using a one-way analysis of variance. A significant treatment effect was seen ($F = 11.21$, $P < 0.01$). Comparisons of individual means using the Newman–Kuels tests revealed that groups b, c, e, f and h were statistically different from the control group ($P < 0.01$).

+ Cytosol progesterin receptor levels in groups a–d, f and g were compared with each other using a one-way analysis of variance. A significant treatment effect was seen ($F = 4.91$, $P < 0.05$). Comparisons of individual means using the Newman–Kuels tests revealed that groups b, c and f were significantly different from the control group ($P < 0.05$).

Vaccination against autoimmune encephalomyelitis with T-lymphocyte line cells reactive against myelin basic protein

Avraham Ben-Nun*, Hartmut Wekerle†
& Irun R. Cohen*

*Department of Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel

†Max-Planck-Institut für Immunbiologie, Freiburg, FRG

Despite differences in initiating events and pathophysiology, the aetiological agents of all autoimmune diseases are lymphocytes specifically reactive against normal constituents of the individual. Recently we have isolated and grown as a cell line rat T lymphocytes reactive against myelin basic protein (BP)¹. This T-cell line originated from rats in which we had induced experimental autoimmune encephalomyelitis (EAE) by immunizing them against BP. Inoculation of syngeneic rats with the T-cell line led to the relatively rapid onset of EAE¹. We report here that attenuation of this cell line provides an agent for establishing resistance to induction of active EAE. Intravenous (i.v.) inoculation of syngeneic rats with cells of the line attenuated by treatment with irradiation or mitomycin C augmented resistance to EAE caused by an encephalitogenic challenge with BP. Thus, aetiological agents of autoimmune disease, like those of microbial disease, when suitably attenuated can be used as effective vaccines.

EAE can be induced in susceptible animals such as rats, guinea pigs, rabbits, monkeys² or man³ by injecting them with BP emulsified in an adjuvant such as complete Freund's adjuvant (CFA). In Lewis rats the disease is characterized by paralysis that is most marked in the tail and hind limbs and which starts usually ~12 days after a single injection of BP in CFA. Histologically the central nervous system shows perivascular infiltrates of mononuclear cells⁴. Unless the rats are aged or have undergone splenectomy or thymectomy⁵ they recover spontaneously from clinical paralysis after a number of days. To study the pathophysiology of EAE we have isolated and propagated *in vitro* a line of Lewis rat T lymphocytes that reacts only against BP, designated Z1a (ref. 1). We found that i.v. inoculation of as few as 10⁵ cells of the Z1a line led to the onset of paralysis in ~4 days. Inoculation of 10⁶ or more cells produced paralysis in ~2–3 days. Most rats recovered from this form of EAE if properly nursed during their paralysis. Table 1 shows the specificity of the proliferative response of the anti-BP Z1a line compared with that of the Z1c line which had been selected for its reactivity against another antigen, the purified protein derivative (PPD) of the mycobacteria present in CFA. The cells of each line responded to its specific antigen, and were also activated by the T-cell mitogen concanavalin A (Con A). Essentially all the cells in both the Z1a and Z1c lines proved to be positive for a T-cell marker using a specific monoclonal antibody (Sera-lab, UK; clone W3/13 HLK) in an immunofluorescence assay⁶.

We investigated the effect of attenuating the Z1a line by inhibiting its cell division. Table 2 shows that i.v. injection of 1 × 10⁷ untreated cells of the Z1a line into syngeneic Lewis rats produced EAE in 18 of 20 rats within 2–3 days. Irradiation of the cells with 1,500 rad or treatment with mitomycin C, agents that block cell division, abrogated the ability of these cells to cause EAE. None of 25 rats that received Z1a cells treated in this way developed EAE. Furthermore, inoculation of

Table 1 Anti-BP and anti-PPD T-cell lines are immunospecific

T-cell line	Proliferative response (c.p.m. × 10 ⁻³ ± s.d.)			
	No antigen	BP	PPD	Con A
Anti-BP (Z1a)	1.7 ± 0.3	48.7 ± 6.1	1.9 ± 0.4	71.4 ± 9.2
Anti-PPD (Z1c)	1.4 ± 0.7	1.2 ± 0.4	77.9 ± 10.4	82.3 ± 12.7

The Z1a and Z1c cell lines originated from the same draining lymph node cell population obtained from female Lewis rats immunized with BP in CFA as described elsewhere¹. To develop the cell lines, Lewis rats were injected in each footpad with 0.05 ml containing BP (25 µg, extracted from guinea pig spinal cords¹⁰ emulsified in equal volumes of phosphate-buffered saline and CFA containing 4 mg ml⁻¹ of *Mycobacterium tuberculosis* H₃₇Ra (Difco). On day 9, the draining lymph nodes were removed and a single-cell suspension prepared. The cells were then selected *in vitro* for BP or PPD by culturing them with either antigen for 72 h. The lymphoblasts that were generated were separated by a discontinuous Ficoll gradient and propagated and maintained *in vitro* as a cell line for several months in medium enriched with T-cell growth factor as reported elsewhere¹. The proliferative responses of the T-cell lines were tested *in vitro* as follows¹. Briefly, 2.5 × 10⁴ cells of either Z1a or Z1c cells were cultured in quadruplicates in flat-bottom microtitre wells in 0.2 ml of Eagle's medium supplemented with 1% fresh autologous rat serum, 2-mercaptoethanol (5 × 10⁻⁵ M), L-glutamine (2 × 10⁻³ M) and antibiotics (streptomycin and penicillin) with added irradiated (1,500 rad) normal syngeneic lymph node cells as accessory cells (5 × 10⁶ cells ml⁻¹) and antigens, BP (50 µg ml⁻¹), or PPD (25 µg ml⁻¹; Statens Serum Institut) or Con A (2.5 µg ml⁻¹; Miles-Yeda, Israel). After 24 h the cultures were pulsed with ³H-thymidine (1 µCi per well, specific activity 10 Ci mmol⁻¹; Nuclear Research Centre, Israel) for 16 h. The cells were then collected on glass fibres using an automatic collector and thymidine incorporation measured in a liquid scintillation counter.

untreated cells of the Z1c line also failed to induce EAE. Thus, induction of EAE is a function of the specific anti-BP Z1a line, a property lost after irradiation or treatment with mitomycin C.

We then tested whether inoculation with cells incapable of inducing EAE could affect the susceptibility of rats to active induction of EAE by later challenge with BP in CFA. Table 3 shows that untreated Lewis rats were highly susceptible to induction of EAE on injection with BP in CFA; 69 of 71 rats developed disease. Intravenous inoculation of cells of the Z1c anti-PPD line, either untreated or irradiated, did not affect this susceptibility and EAE was induced in all 20 rats challenged with BP in CFA. In contrast, a single i.v. injection of 1 × 10⁷ Z1a cells attenuated by treatment with mitomycin C or irradiation led to significantly increased resistance to induction of EAE. Only 14 of a total of 40 rats showed any signs of paralysis and the degree of the paralysis in these rats was judged to be much milder than that appearing in the other groups. Thus it seems that vaccination with attenuated autoimmune T lymphocytes

Table 2 Attenuated T lymphocytes of the Z1a anti-BP line do not produce EAE

Line	Inoculation of T-cell lines	
	Treatment	Incidence of EAE
Anti-BP (Z1a)	Untreated	18/20
	Irradiated	0/15
	Mitomycin C	0/10
Anti-PPD (Z1c)	Untreated	0/20

Healthy female Lewis rats (2–3 months old) were injected i.v. with 1 × 10⁷ cells of T-lymphoblast cell lines specifically reactive against BP (Z1a) or PPD (Z1c). Before inoculating the cell lines into normal syngeneic animals, they were re-stimulated *in vitro* with the relevant antigen, in the presence of irradiated (1,500 rad) syngeneic accessory cells for 72 h (ref. 1). The cells, >80% lymphoblasts, were then collected and injected, either untreated or attenuated by irradiation (1,500 rad) from a ⁶⁰Co source, or treatment with mitomycin C (50 µg per 10⁷ cells per ml; Sigma) at 37°C for 40 min. The treated cells were washed extensively before being inoculated. EAE was diagnosed clinically by overt paralysis of the hind limbs and histologically by perivascular mononuclear cell infiltration of the central nervous system¹.

Table 3 Attenuated anti-BP T-cell line vaccinates rats against induction of EAE

Vaccination with T-cell line	Incidence of EAE in response to injection of BP in CFA	% Inhibition of EAE
None	69/71	—
Anti-PPD (Z1c)		
Untreated	10/10	0
Irradiated	10/10	0
Anti-BP (Z1a)		
Irradiated	8/25*	68
Mitomycin C	6/15*	60

EAE was induced in naive Lewis rats (2–3 months old) or in animals that had been vaccinated i.v. 3 weeks earlier with 10^7 cells of the anti-PPD Z1c T-cell line or with cells of the anti-BP Z1a T-cell line that were either irradiated (1,500 rad) or treated with mitomycin C, as described in Table 2 legend. EAE was induced by injecting BP in CFA into the hind footpads of the animals, as described in Table 1 legend.

* $P < 0.001$.

provided protection against active EAE for about 65% of the rats.

We do not know the mechanism by which the attenuated T lymphocytes increased resistance to induction of EAE; however, it seems reasonable to suspect that some process of immunity was involved. The Z1a anti-BP lymphocytes probably differed from the ineffective Z1c anti-PPD lymphocytes in the structure of their antigen receptors (Table 1). Antigen receptors of T lymphocytes⁷ as well as of B lymphocytes or antibodies⁸ can be immunogenic. Immunity against antigen receptors, anti-idiotypic immunity, has been proposed to serve as a mechanism that regulates immune responses by suppressing or activating specific clones of lymphocytes bearing the target receptors⁹. Vaccination with attenuated Z1a cells might have produced an immune response against endogenous clones of lymphocytes with anti-BP receptors. As anti-BP clones are the aetiological agents of EAE, development of the disease would be inhibited. Therefore, our results could be explained by anti-receptor immunity raised against the autoimmune lymphocytes that mediate EAE. However, other explanations are possible and the anti-receptor hypothesis must be tested experimentally.

Whatever the mechanism of protection, the procedure described here can be conceptually related to vaccination against infectious diseases in which inoculation of an attenuated agent of disease induces a degree of protection against the virulent pathogen. In the case of autoimmunity, the aetiological agent of disease is not a microbe, but arises within the immune system of the individual. Our results indicate that an artificially induced autoimmune disease may be mitigated or prevented by vaccination against specific effector lymphocytes. A different but related problem is posed by the need to treat the spontaneous, often chronic processes that characterize the important autoimmune diseases of man.

We thank Mr H. Otmy for technical assistance and Professor M. Feldman for his support. This study was supported by a grant from the Stiftung Volkswagenwerk, Hannover.

Received 3 February; accepted 30 March 1981.

- Ben-Nun, A., Wekerle, H. & Cohen, I. R. *Eur. J. Immun.* **11**, 195–199 (1981).
- Paterson, P. Y. in *Autoimmunity, Genetic, Immunology, Virology and Clinical Aspects* (ed. Talal, N.) 664–692 (Academic, New York, 1977).
- Paterson, P. Y. in *Immunological Disease* 3rd edn (eds Samter, M. et al.) 1400–1435 (Little, Brown and Co., Boston, 1978).
- Paterson, P. Y., Drobish, D. G., Hanson, M. A. & Jacobs, A. F. *Int. Archs Allergy appl. Immun.* **37**, 26–40 (1970).
- Ben-Nun, A., Ron, J. & Cohen, I. R. *Nature* **288**, 389–390 (1980).
- Ben-Nun, A., Wekerle, H. & Cohen, I. R. (in preparation).
- Binz, H. & Wigzell, H. *J. exp. Med.* **144**, 1438–1457 (1976).
- Hämmerling, G., Bleck, S. J., Berek, C., Eichmann, K. & Rajewski, K. *J. exp. Med.* **143**, 861–869 (1976).
- Jerne, H. K. *Annls Immun. Inst. Pasteur, Paris* **125**, 373–389 (1974).
- Hirshfeld, H., Teitelbaum, D., Arnon, R. & Sela, M. *FEBS Lett.* **7**, 317–320 (1970).

C-terminal sequence of the secreted form of mouse IgD heavy chain

Renate Dildrop & Konrad Beyreuther

Institut für Genetik der Universität zu Köln, D-5000 Köln, 41, FRG

Immunoglobulins have been identified as membrane-bound molecules on the surface of B lymphocytes and as secreted products of plasma cells. In the case of immunoglobulin M (IgM) the carboxy-terminal sequences of the μ -chains of membrane-bound and secreted molecules differ from each other and are encoded by different exons of the μ constant region ($C\mu$) gene. The coding sequence for the C-terminus of the secreted μ -chain is contiguous with the 3' end of the $C\mu 4$ exon and separate exons downstream of $C\mu 4$ encode the C-terminus of the membrane-bound chain^{1–3}. Immunoglobulin D is also found membrane-bound and as a secreted molecule, and recent data indicate that the exon arrangement of the $C\delta$ gene is in part similar to that of the $C\mu$ gene^{4,5}. However, the amino acid sequence analysis presented here demonstrates that in the case of IgD the C-terminus of the secreted δ -chain is encoded by a separate exon (the $C\delta DC$ exon of Tucker *et al.*⁵) and not by the $C\delta AC$ sequence which corresponds topographically to the sequence expressed at the C-terminus of secreted μ chains.

The cell line B1-8.81 (IgD, $\lambda 1$) has been isolated as a switch variant of the cell line B1-8.64.1 (IgM, $\lambda 1$) and is of C57BL/6 origin⁶. It secretes a monoclonal IgD antibody with specificities for the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP). Anti-NP antibodies from B1-8.81 ascites fluid were purified by affinity chromatography⁶. The heavy chains of these molecules are linked to each other and to the light chains by disulphide bridges (ref. 6 and unpublished data). After complete reduction and carboxyamidomethylation the heavy and light chains were eluted from Sephadex G-100 with 4.5 M urea, 1 M propionic

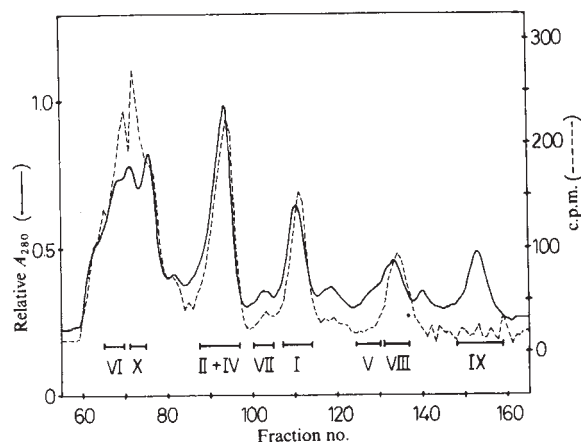


Fig. 1 Gel filtration of cyanogen bromide-cleaved peptides of ^{14}C -carboxyamidomethylated B1-8.81 heavy chain. Cyanogen bromide cleavage of 50 mg of the completely reduced and alkylated heavy chain was performed in 70% formic acid at a concentration of 10 mg ml^{-1} with a fivefold excess (w/w) of CNBr for 24 h at 20°C . The mixture was dried under a stream of nitrogen. The peptides were dissolved in 3.5 ml of 0.1 M formic acid containing 6 M deionized urea and applied to a Sephadex G-50 superfine column ($2 \times 200 \text{ cm}$) equilibrated in the same solvent. Fractions of 3.3 ml were collected and aliquots of $20 \mu\text{l}$ were used for liquid scintillation counting in 5 ml of Bray's solution¹⁰. Roman numerals of the pooled fractions refer to the position of the corresponding peptides in the sequence of the B1-8.81 heavy chain as given in Fig. 2. Peptide no. III was insoluble in 6 M urea, 0.1 M formic acid, and could thus be isolated without chromatographic separation.

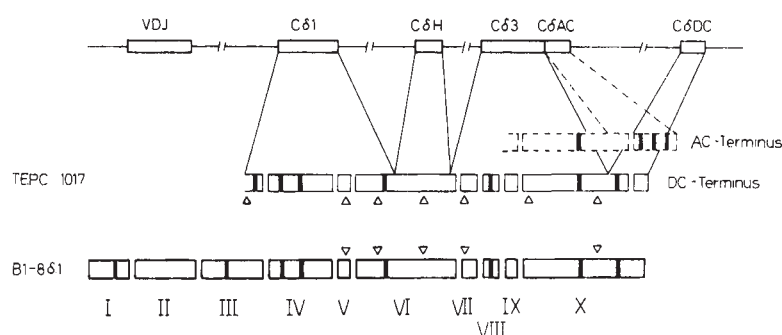


Fig. 2 Schematic representation of the CNBr-cleaved peptides in the sequence of the secreted B1-8.δ1 heavy chain. The genomic organization of the Cδ gene according to Tucker *et al.*⁵ is given at the top of the figure. The translated TEPC 1017 cDNA sequence is schematically represented by CNBr-peptides (open boxes). The positions of cysteines (black bars) and of putative carbohydrate attachment sites (open arrow heads) are indicated. Hypothetical CNBr-fragments corresponding to a chain which would terminate with the AC-segment are shown as dashed lines. The alignment of the CNBr-fragments of the B1-8.δ1 heavy chain is given in the lower part of the figure. Alignment of peptides I–III is according to Bothwell *et al.*¹¹. No carbohydrate was found in peptide III and in the N-terminal tryptic peptide of X. Peptide III contains only one cysteine.

acid. The isolated heavy chains were treated with cyanogen bromide and peptides were separated by column chromatography on Sephadex G-50 (Fig. 1). Column fractions containing more than one peptide were rechromatographed on either Sephadex G-100 or Sephadex G-50. The purified CNBr fragments of the δ heavy chain were characterized by end group and compositional analysis and carbohydrate and cysteine contents (cysteines were labelled with ¹⁴C-iodoacetamide during reduction and alkylation, carbohydrate was detected as glucosamine in amino acid analysis).

The published IgD heavy-chain DNA sequence⁵ enabled us to identify all constant-region CNBr fragments. They correspond to the Cδ1, CδH and Cδ3 exons as defined by Tucker *et al.* (Fig. 2). Five of the seven predicted⁵ carbohydrate attachment sites carry sugar, except the first proposed site in Cδ1 and the second one in Cδ3. The lack of carbohydrate in the first proposed site in Cδ1 can be deduced from the absence of carbohydrate from CNBr fragment III of the B1-8.δ1 chain. It is known that the sequence asparagine–phenylalanine–threonine is not necessarily used as a carbohydrate attachment site⁷. However, our determination of radioactive iodoacetamide incorporation (data not shown) indicates that the CNBr fragment III also does not contain the cysteine residue expected in position 5 of Cδ1 (Fig. 2). If the 5' intron–exon junction of Cδ1 were defined not by the third RNA splice site proposed by Tucker *et al.*⁵ but by the fourth site situated 22 nucleotides further downstream 1 the Cδ1 domain would be shortened by seven amino acids and lack both cysteine and carbohydrate attachment site. Alternatively, the lack of these two structures in the B1-8.δ1 CNBr fragment III could reflect an allotypic difference between the δ-chains from C57BL/6 and those of BALB/c from which the TEPC 1017 tumour⁵ originates.

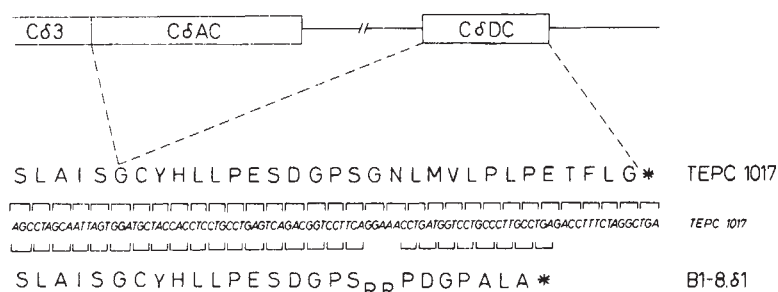
The protein sequence of the second carbohydrate attachment site in Cδ3 is conserved in the B1-8.δ1 heavy chain, but not used for carbohydrate attachment. Neither the predicted C-terminal CNBr fragment of the AC-gene segment nor that of the DC-exon was identified (Fig. 2). This can be explained by a base pair exchange in the corresponding methionine codon, representing a sequence difference between B1-8.δ1 and TEPC 1017 δ-

chains. If the AC-gene segment were expressed in the secreted form of the δ heavy chain the loss of the cyanogen bromide cleavage site would result in a new CNBr fragment containing four cysteines. By comparing the amount of incorporated radioactivity with that of other cysteine-containing peptides, the CNBr fragment X, the only candidate for the C-terminus, was found to contain only two cysteines. This would be expected if the DC-segment is expressed in the secreted B1-8.δ1 heavy chain.

Tryptic peptides of this CNBr fragment were separated on Sephadex G-50 in 0.5 M formic acid and further analysed by automated Edman degradation. The sequence of the C-terminal 27 amino acids is presented in Fig. 3. The sequence data clearly show that the C-terminus of the secreted B1-8.δ1 heavy chain is encoded by the CδDC exon. The sequence of the C-terminus is identical to that predicted by the sequence of the TEPC 1017 cDNA in the C-terminal 5 amino acid positions of Cδ3 and the first 13 positions of CδDC. The last nine residues of the B1-8.δ1 heavy chain do not fit the predicted sequence at all. This problem can be resolved, however, by exchanging in the TEPC 1017 DNA a single base pair and inserting another one (note that both changes occur in the upper part of a possible hairpin structure). The resulting frameshift generates the B1-8.δ1 C-terminal heavy-chain sequence.

It is now clear that the difference between the cDNA-derived TEPC 1017 C-terminal δ-chain sequence and the sequence of the B1-8.δ1 chain C-terminus is not due to genetic polymorphism. Tucker *et al.*⁸ have very recently isolated the CδDC exon from BALB/c liver DNA and found that it encodes an amino acid sequence identical to that expressed in the C-terminus of the B1-8.δ1 chain. Thus, the TEPC 1017 tumour line has either accumulated somatic mutations in the Cδ gene or, more likely, the difference between cDNA and germ-line gene sequences is due to errors in DNA–RNA or RNA–DNA transcription. Taken together, our results show that the C-terminus of the secreted δ-chain is encoded by the CδDC exon and that CδAC-encoded sequences are not expressed in the secreted δ-chain. B1-8.δ1 cells also carry IgD on the surface and the surface-bound δ-chains have a higher molecular weight than the

Fig. 3 Sequence of the C-terminal part of secreted B1-8.δ1 heavy chain. The C-terminal sequence of IgD heavy chains from TEPC 1017 and B1-8.δ1 are aligned to the corresponding TEPC 1017 exons and cDNA sequence. The TEPC 1017 sequence was taken from ref. 5. The B1-8.δ1 sequence was obtained by automated Edman degradation of tryptic peptides using an updated Beckman 890 B sequencer. Degradations were performed in the presence of 3 mg of the non-protein carrier Polybrene¹² using a 0.2 M Quadral program¹³. Phenylthiohydantoin derivatives obtained after conversion using the Sequemat P6 autoconverter were identified by HPLC¹⁴ and TLC¹⁵. The C-terminal nine amino acids of B1-8.δ1 are different from the TEPC 1017 cDNA sequence but not from the sequence deduced from the germ-line DNA⁸. The cDNA and germ-line DNA sequence differ by one inserted and one exchanged nucleotide. A key to the single letter amino acid code is provided by Dayhoff¹⁶.



secreted ones⁶. It seems likely that, as in the case of IgM, the C-termini of the two chains differ from each other and an as yet unidentified exon encodes the C-terminus of the surface-bound δ -chain.

This work was initiated by M. S. Neuberger who also supplied the B1-8.81 protein. We thank K. Rajewsky for stimulating discussions during the work and preparation of the manuscript, and G. Zimmer and K. Neifer for technical assistance. This work was supported by the Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen through a Forschungsbeihilfe to K.B. and K.R. and by the Deutsche Forschungsgemeinschaft through SFB 74 to K.B.

Received 12 February; accepted 28 April 1981.

1. Singer, P. A., Singer, H. H. & Williamson, A. R. *Nature* **285**, 294–300 (1980).
2. Rogers, J. et al. *Cell* **20**, 303–312 (1980).
3. Early, P. et al. *Cell* **20**, 313–319 (1980).
4. Liu, C., Tucker, P. W., Mushinski, J. F. & Blattner, F. R. *Science* **209**, 1348–1352 (1980).
5. Tucker, P. W., Liu, C., Mushinski, J. F. & Blattner, F. R. *Science* **209**, 1353–1360 (1980).
6. Neuberger, M. S. & Rajewsky, K. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1138–1142 (1981).
7. Kehry, M., Sibley, C., Fuhrman, J., Schilling, J. & Hood, L. E. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2932–2936 (1979).
8. Tucker, P. W. et al. in *The Lymphocytes in the Immune Response* (eds Mosier, D., Klinman, N., Sher, E. & Vitetta, E.) (Elsevier, Amsterdam, in the press).
9. Waxdal, M. J., Konigsberg, W. H., Henley, W. L. & Edelman, G. M. *Biochemistry* **7**, 1959–1966 (1968).
10. Bray, G. *Analyt. Biochem.* **1**, 279–285 (1960).
11. Bothwell, A. L. M. et al. *Cell* (in the press).
12. Tarr, G. E., Beecher, J. F., Bell, M. & McKean, D. J. *Analyt. Biochem.* **84**, 622–627 (1978).
13. Beyreuther, K., Raufuss, H., Schrecker, O. & Hengstenberg, W. *Eur. J. Biochem.* **75**, 275–286 (1977).
14. Johnson, N. D., Hunkapiller, M. W. & Hood, L. E. *Analyt. Biochem.* **100**, 335–338 (1979).
15. Beyreuther, K. in *Solid Phase Methods in Protein Sequence Analysis*, 107–119 (Elsevier, Amsterdam, 1977).
16. Dayhoff, M. *Atlas of Protein Sequence and Structure* Vol. 5, D2 (National Biomedical Research Foundation, Washington, 1972).

SV40 T antigen binds specifically to a cellular 53 K protein *in vitro*

Frank McCormick*, Robin Clark†, Ed Harlow* & Robert Tjian†

* Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK

† Department of Biochemistry, University of California, Berkeley, California 94720, USA

Simian virus 40 (SV40) large T antigen is a multifunctional protein that is essential for virus replication and the establishment and maintenance of cell transformation¹. The molecular basis of these functions probably involves specific interactions of T antigen with DNA^{2–4} and cellular proteins. An example of such an interaction has been described recently; a significant fraction of the T antigen extracted from cell lysates is tightly bound to a cellular phosphoprotein of molecular weight 53,000 (53K)^{5–9}. Here we demonstrate *in vitro* the direct interaction of highly purified T antigen (D2 T antigen or SV80 T antigen) with 53K in lysates from a variety of mouse cell lines. All the 53K detected in these lysates was able to bind to added T antigen, and it was the only cellular protein with which T antigen formed a stable complex. We conclude that 53K binds directly to a specific site on T antigen and that binding occurs without previous modification and without involvement of other virus-encoded or induced proteins.

The function of 53K is unknown, but several interesting properties have been attributed to this protein in addition to its association with large T antigen. Transformed cells seem to express significantly higher levels of 53K than normal cells^{6,10}. Similarly, SV40-infected mouse or monkey cells synthesize 53K more rapidly than uninfected cells^{9,11}. Furthermore, lymphocytes freshly prepared from the spleens of young mice express

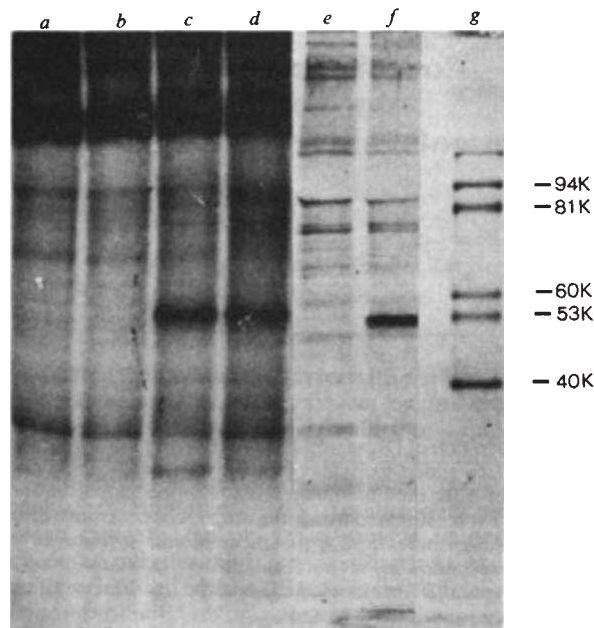


Fig. 1 Binding of purified D2 to 53K *in vitro*. F9 mouse embryonal carcinoma cells (supplied by P. Goodfellow) were labelled with ³²P-labelled inorganic phosphate (1 mCi ml⁻¹ phosphate-free medium) or ³⁵S-Met (1 mCi ml⁻¹ Met-free medium) as described elsewhere⁷. Monolayers were washed with phosphate-buffered saline and cells lysed in buffer A (2% NP-40, 10 mM Tris, 140 mM NaCl, pH 8.0; 4 °C for 15 min). These lysates were centrifuged for 2 min in an Eppendorf microfuge and the supernatants used as a source of labelled 53K. D2 protein (1 µg of purified protein) were added to lysates corresponding to ~2 × 10⁵ F9 cells in 200 µl extraction buffer. After incubation at room temperature for 15 min, 5 µl of preimmune rabbit serum was added and incubated at 4 °C overnight. Rabbit immunoglobulin G (IgG) was removed with 50 µl of 10% fixed *Staphylococcus aureus*. The remaining solution was immunoprecipitated and immune complexes were washed twice in buffer A and twice in 2 M urea, 0.4 M LiCl, 10 mM Tris, pH 8.0, and subjected to SDS-polyacrylamide gel electrophoresis as described elsewhere¹⁸. Tracks a–d are from cells labelled with ³²P-phosphate; tracks e–f are from ³⁵S-labelled cells. Track g is a molecular weight marker mix. a, 5 µl anti-D2 serum; b and e, 5 µl anti-D2 serum; c and f, 5 µl anti-D2 serum in the presence of 1 µg D2 protein; d, anti-53K serum F5 (ref. 7).

detectable levels of 53K only after mitogenic stimulation with an agent such as concanavalin A¹². Another interesting observation is that some mice bearing SV40-induced tumours raise autoantibodies against 53K^{5,8}. Because 53K synthesized by SV40-transformed or infected cells becomes complexed with large T we are interested in determining how these proteins interact and whether this interaction relates to the role of large T in promoting DNA synthesis^{13,14}, regulating early mRNA synthesis^{15–17} and in cellular transformation (see discussion in ref. 1).

Here we describe investigations of the mechanism of binding of SV40 large T with 53K in an *in vitro* system. We have used the adenovirus: SV40 hybrid protein D2 as a convenient source of T antigen and have assayed for the ability of purified D2 protein to bind radiolabelled 53K in detergent lysates from various cells. In early experiments, 3T6 cells were used. However, the F9 line of embryonal carcinoma cells seems to express significantly more 53K and was therefore used here.

Purified D2 protein was added to lysates of labelled F9 cells and incubated at room temperature for 15 min. It was then immunoprecipitated from these lysates using antiserum raised against purified D2 protein or with monoclonal antibodies which recognize a single determinant on the protein. It is clear from Fig. 1 that anti-D2 antibodies immunoprecipitated a 53K phosphoprotein from those lysates to which D2 protein had

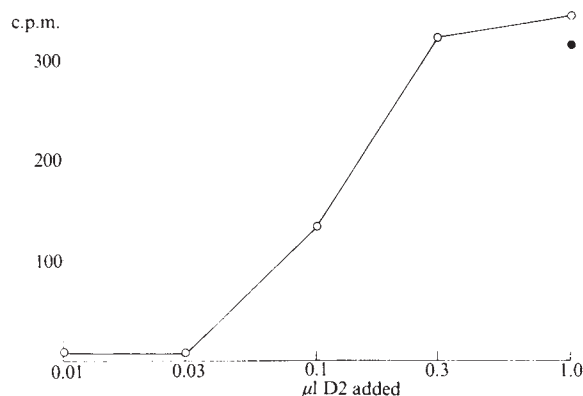


Fig. 2 Titration of D2 against 53K. Various concentrations of D2 protein were added to lysates of ^{35}S -Met-labelled F9 cells. The amount of 53K immunoprecipitated by monoclonal antibodies against D2 was estimated by cutting the 53K bands from a dried gel and counting them in a liquid scintillation system. In these immunoprecipitations, monoclonal antibodies which recognize a determinant on D2 protein were used. The hybridoma cells which produce these antibodies were obtained by fusing spleen cells from BALB/c mice bearing SV40-induced tumours with the myeloma line P-3-NS1-1Ag-4-1. These mice had been given an injection of partially purified T antigen/53K complex 3 days before spleen removal. Details of this and other hybridoma cell lines will be described elsewhere¹⁹. One of these lines, L21, produces antibodies directed against 53K¹⁹.

been added. In the absence of added D2 protein, no cellular proteins were precipitated specifically. Similar results were obtained with T antigen purified from SV80 cells. These results suggest that the added T antigen binds to labelled 53K in these lysates and thus allows its immunoprecipitation by antisera that have no detectable activity against 53K itself. The 53K protein that seemed to bind to T antigen was not released during stringent washing of immune complexes with buffers containing 0.5% NP-40, 0.4 M LiCl or 2 M urea. In these conditions, no other labelled cellular proteins were immunoprecipitated specifically in association with T antigen. Thus the complex

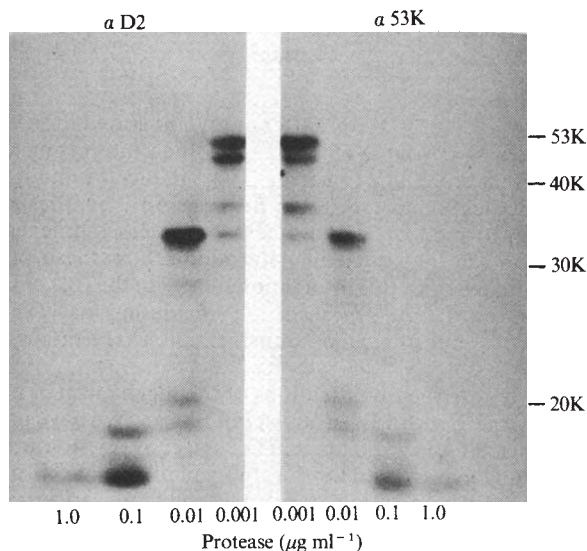


Fig. 3 V8 protease digestion of 53K. 53K was immunoprecipitated directly with monoclonal antibodies against 53K (from hybridoma clone L21) or indirectly by monoclonal antibodies which recognize D2 (clone L7) in the presence of added D2 protein. Immunoprecipitates were treated as described in Fig. 1 legend and the 53K bands removed from gels electrophoretically. Aliquots of the labelled protein were digested with various concentrations of V8 protease and again subjected to SDS-PAGE¹⁸.

formed *in vitro* resembled that extracted from SV40-transformed 3T3 cells⁷. We can also conclude that the polypeptide sequences which large T shares with small t are not required for binding, as these sequences are not represented on D2 protein, and that the mutation on SV80 T antigen which destroys its ability to initiate SV40 DNA synthesis (Gish and Botchan, personal communication) does not affect its ability to bind 53K.

When increasing concentrations of D2 protein were added to a constant volume of F9 cell lysate, the amount of 53K immunoprecipitated by anti-D2 antibodies was proportional to the amount of D2 T antigen added (Fig. 2). The maximum amount of 53K that could be detected by this indirect immunoprecipitation assay corresponded closely to the maximum amount that could be immunoprecipitated directly by a monoclonal antibody against 53K. Thus, all the immunologically reactive 53K from these cell lysates seemed to be available for binding to added D2.

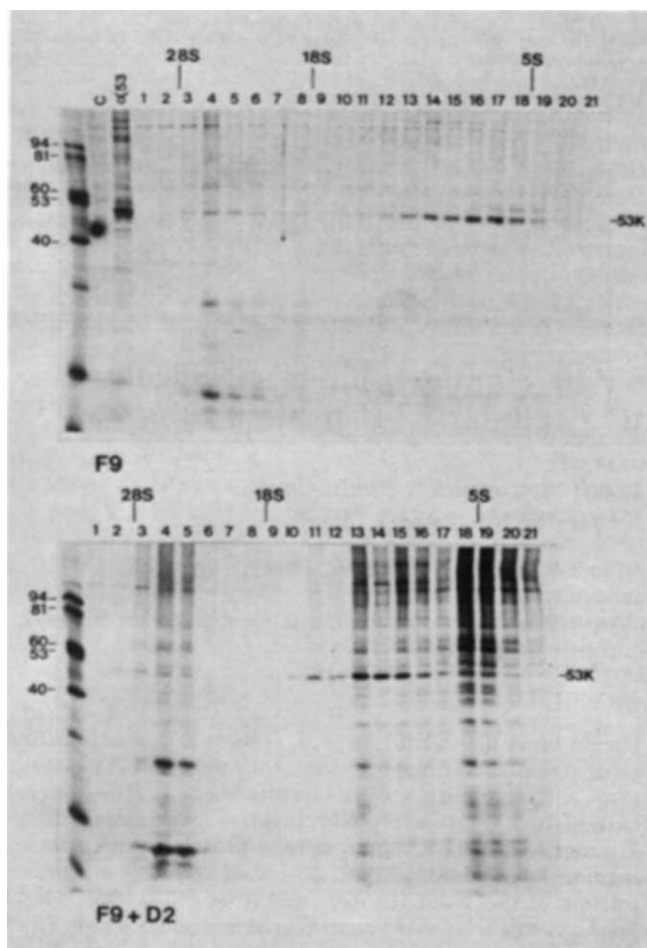


Fig. 4 Sedimentation of 53K in the presence of added D2 protein. F9 cells were labelled with ^{35}S -Met (0.24 mCi ml^{-1} , 2 h) and lysates from these cells were incubated at 22°C in the presence (lower panel) or absence (upper panel) of D2 protein ($2 \mu\text{g per ml}$ lysate). Each lysate was centrifuged at 25,000 r.p.m. through sucrose gradients as described elsewhere⁷ and each fraction was immunoprecipitated with $10 \mu\text{l}$ L21 anti-53K hybridoma supernatant. Immune complexes were treated as in Fig. 1 legend. Tract C in the top panel represents an immunoprecipitation of unfractionated, untreated F9 lysate with anti-alkaline phosphatase monoclonal antibodies. Track 53K represents immunoprecipitation of the same lysate with anti-53K antibodies.

The 53K species immunoprecipitated directly by antibodies was compared with the 53K species that formed a stable complex with D2 protein. Figure 3 shows that the products of partial proteolysis generated by addition of V8 protease were identical for the two 53K samples, which suggests that these are closely related if not identical, proteins.

We have done similar *in vitro* binding assays using lysates from a variety of cell lines: these include 3T3 cells transformed by polyoma virus⁵, 3T6 cells infected with wild-type polyoma virus, mouse L-cells and 3T6 cells. In each case, a single polypeptide of molecular weight (MW) 53,000 was immunoprecipitated by anti-D2 sera after addition of T antigen to the respective lysates (data not shown). The availability of 53K for binding in these cell lysates suggests that other proteins in the lysates, for example, polyoma virus T antigen, do not compete effectively for binding with SV40 large T.

The data described here provides direct evidence that purified antigen added to lysates of cells expressing 53K is able to form a complex with this protein. We have further demonstrated this association by determining the sedimentation properties of 53K in the presence and absence of D2 protein. Figure 4 shows that most of the 53K extracted from F9 cells sediments at ~8S, corresponding to a molecular weight of the order of 200,000. This could represent a tetrameric structure, or an association of 53K with other macromolecules. A small fraction of the 53K from these cells sediments at 20S, which suggests a native molecular weight of up to 1 million. It is unclear whether this represents homopolymers of 53K or a high-molecular-weight complex which contains other components. After addition of D2 protein to lysates of F9 cells, the lower-molecular-weight form shifted to a sedimentation value of 12S, which corresponds to a native molecular weight of ~300,000. Similar results were obtained when either anti-53K or anti-D2 antibodies were used to immunoprecipitate the complex. These results suggest that one molecule of D2 protein binds to a 200,000-MW form of 53K or these conditions. It should be stressed, however, that these sedimentation values cannot be used to determine molecular weights with a high degree of reliability, as the sedimentation properties of proteins are affected by their configuration and gradients of the type described here do not provide high resolution. Nevertheless, it is clear that the addition of D2 protein to lysates of F9 results in a significant increase in the molecular weight of 53K in its native state which indicates a physical association of the two protein species.

We have been able to demonstrate the binding of SV40 T antigen to a cellular 53K protein *in vitro*, by two independent methods. The development of this system should allow us to assess whether binding of 53K affects the biochemical activity of T antigen and thus modifies its biological functions.

We thank D. Lane for anti-D2 serum and Michael van Wilde, Kit Osborne and Alan Robbins for growth of cells. During part of this work study, F. M. held an ICRETT Travel Fellowship from UICC, Geneva. E. H. is a fellow of the Lady Tata Memorial Trust.

Received 4 December 1980; accepted 29 April 1981.

1. Tooze, J. in *Molecular Biology of Tumor Viruses*, Part 2 (ed. Tooze, J.) (Cold Spring Harbor Laboratory, New York, 1980).
2. Tjian, R. *Cell* **13**, 165–179 (1978).
3. Shortle, D. R., Margolske, R. F. & Nathans, D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6128–6131 (1979).
4. Myers, R. M. & Tjian, R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6491–6495 (1980).
5. Lane, D. P. & Crawford, L. V. *Nature* **278**, 261–263 (1979).
6. Linzer, D. I. H. & Levine, A. J. *Cell* **17**, 43–52 (1979).
7. McCormick, F. & Harlow, E. *J. Virol.* **34**, 213–224 (1980).
8. Gurney, E. G., Harrison, R. O. & Fenno, J. J. *J. Virol.* **34**, 752–763 (1980).
9. Harlow, E., Pim, D. C. & Crawford, L. V. *J. Virol.* **37**, 564–573 (1981).
10. Crawford, L. V., Pim, D. C., Gurney, E. G., Goodfellow, P. & Taylor-Papadimitriou, J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 41–45 (1981).
11. Linzer, D. I. H., Maltzman, W. & Levine, A. J. *Virology* **48**, 303–318 (1979).
12. Milner, J. & McCormick, F. *Cell Biol. int. Rep.* **4**, 663–669 (1980).
13. Tegtmeyer, P. *J. Virol.* **10**, 591–598 (1972).
14. Tjian, R., Fey, G. & Graessman, A. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1279–1283 (1978).
15. Tegtmeyer, P., Schwartz, M., Collins, J. K. & Rundell, K. J. *J. Virol.* **16**, 168–178 (1975).
16. Reed, S. I., Stark, G. R. & Alwine, J. C. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1605–1609 (1976).
17. Rio, D., Robbins, A., Myers, R. & Tjian, R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5706–5710 (1980).
18. Smith, A. E., Smith, R. & Paucha, E. *J. Virol.* **28**, 140–149 (1978).
19. Harlow, E., Crawford, L., Pim, D. & Williamson, N. *J. Virol.* (in the press).

Persistence and expression of histone genes injected into *Xenopus* eggs in early development

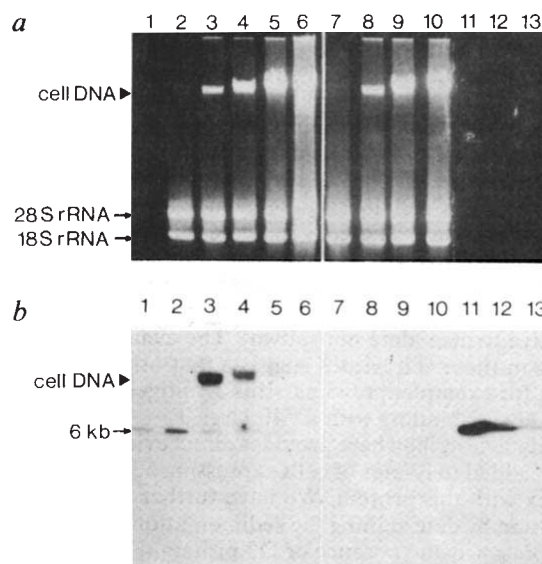
Mary M. Bendig

Institut für Molekularbiologie II der Universität Zürich, Hönggerberg, 8093 Zürich, Switzerland

Microinjection of amphibian oocytes with purified cloned DNA has proven to be a valuable assay system for studying eukaryotic gene expression^{1–13}. The oocyte's ability to express microinjected genes has already allowed a functional analysis of several genes and their sequence manipulated counterparts^{14–20}. Although such studies are now possible with several other expression systems, the oocyte system is unique in that it represents a cell destined, on fertilization, to divide and differentiate into an embryo. Thus, it was envisaged that the technique of microinjecting genes and their mutant analogues into fertilized eggs might eventually be used as an assay system for studying the transcriptional control processes that occur during cell differentiation and development. To this end, fertilized eggs of *Xenopus laevis* were injected with a cloned repeat of sea urchin histone genes and the fate and expression of the injected genes examined during the early stages of *Xenopus* development. After replication during the early cleavage stages, the injected sea urchin histone DNA sequences persisted to at least the swimming tadpole stage. The injected genes were also at least in part faithfully transcribed into RNA species with the correct histone mRNA 5' and 3' termini. Thus, these results, reported here, meet the prerequisites for an assay system capable of being used to investigate the factors involved in the developmental control of gene activity.

Fertilized eggs were injected with sea urchin histone DNA in one of two forms—as a linear 6-kilobase (kb) *Hind*III fragment recovered from the recombinant h22 (ref. 21) or as supercoiled, circular pBR322 recombinant plasmid DNA containing two copies of the 6-kb *Hind*III fragment tandemly integrated head to tail. The h22 repeat contains all five histone genes and transcription of all five proceeds in the same direction²². Nucleic acid was isolated from the developing embryos at various stages post-injection and the persistence and physical state of the injected DNA sequences were analysed by blot hybridization. In Figs 1 and 2, each gel slot was loaded with a sample representing one embryo equivalent of nucleic acid isolated at the indicated stage of development. The hybridization probe was ³²P-labelled pBR322 recombinant plasmid DNA containing the coding region of the sea urchin H1 histone gene. This H1 probe was chosen because, in contrast to the entire repeat, it gave no background hybridization to nucleic acid samples prepared from uninjected embryos (Fig. 1, lanes 7–10). Clear hybridization is seen in developing embryos after injection of either linear (Fig. 1) or circular DNA (Fig. 2). Moreover, the injected DNA sequences were replicated in the early stages of development, consistent with, and extending, the recent report that DNA injected into unfertilized eggs supports semiconservative replication of the injected DNA²³. Thus, by comparing lanes 2 and 3 in Figs 1 and 2 with known amounts of DNA (Fig. 1, lanes 11–13; 2,500, 250 and 25 pg, respectively), clearly there is at least a 10-fold increase in the amount of hybridizable sequences in the embryo at late blastula as compared with the amount in the egg immediately following injection. As the embryos developed further, the amount of hybridizable DNA per embryo steadily decreased, presumably reflecting selective degradation of the injected DNA (Figs 1, 2, lanes 3–6). However, faint amounts of injected DNA sequences were detectable as late as the swimming tadpole stage following injection of linear DNA (Fig. 1, lane 6) and as late as the early

Fig. 1 Analysis by DNA blotting of the persistence and physical state of injected DNA sequences in developing embryos of *X. laevis* following the injection of fertilized eggs with linear molecules of sea urchin histone DNA. Artificially fertilized eggs of *X. laevis* were prepared as described by Billet and Wild²⁹. Mature male and female frogs received two priming doses of Pregnyl (Organon). Eggs were squeezed out through the cloaca and fertilized immediately with a freshly prepared suspension of sperm. After chemical decapsulation with papain, healthy, fertilized eggs were injected within 1 h, before the first cleavage event occurred. The microinjection technique was essentially the same as used for oocytes^{1,2}, except that in the fertilized egg the germinal vesicle has disappeared and direct nuclear injection is not necessary. A linear 6-kb *Hind*III fragment containing the histone gene repeat of *P. miliaris* was recovered from the recombinant λ h22 (ref. 21) and purified by actinomycin/CsCl gradient centrifugation. In most experiments, 250 pg of DNA in a volume of 10 nl was injected per egg. Eggs injected with 500 pg of DNA did not develop beyond the late blastula stage. According to previous reports, however, even after injection of 2–4 ng of DNA, 15% of the embryos reached the feeding tadpole stage³⁰. About 2 h after injection, embryos, usually now at the four-cell cleavage stage, were transferred from Barth's medium to sterile 'aged' water plus antibiotics (Gibco) and the developing embryos incubated at 18 °C. Samples of 5–10 embryos were collected immediately following injection and at the indicated stages of development³¹ up to and including the swimming tadpole. Embryos were homogenized at 0 °C in 10 mM EDTA, 100 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1 mg ml⁻¹ proteinase K and 2% w/v SDS. After a 30-min incubation at room temperature, the homogenate was phenol-extracted three times, chloroform-extracted twice and the nucleic acids precipitated with two volumes of ethanol. The equivalent of one egg or embryo's worth of nucleic acid isolated from progressive stages of development was electrophoresed through a 0.8% agarose gel. The gel was stained with ethidium bromide and photographed (panel a). To obtain efficient blotting of supercoiled, circular DNAs and large molecular weight DNAs, the gel was treated for 10 min with 0.25 M HCl before denaturation with 0.5 M NaOH, neutralization and transfer to nitrocellulose filters^{32,33}. The transferred DNA was hybridized to a ³²P-nick-translated probe of a pBR322 recombinant plasmid containing the ~500-bp coding region of the H1 histone gene of the h22 repeat (as constructed and provided by M. Busslinger). Autoradiography was performed with an intensifier screen at -70 °C (panel b). Lane 1 is a control sample taken from the loaded injection needle before egg injection and represents the amount of DNA injected per egg. Lanes 2–6 represent nucleic acid samples prepared from egg/embryos at various developmental stages following DNA injection, and lanes 7–10 represent similarly prepared samples from uninjected embryos: lanes 2, 7, stage 1 (egg); lanes 3, 8, stages 8–9 (late blastula); lanes 4, 9, stages 21–23 (early tail-bud); lanes 5, 10, stages 35–36 (hatching); lane 6, stage 42 (swimming tadpole). Lanes 11, 12 and 13 are quantitative controls containing 2,500, 250 and 25 pg, respectively, of the h22 6-kb linear DNA used for injection.



tail-bud stage following injection of circular DNA (Fig. 2, lane 5).

The results of these experiments also show that the physical form of the injected DNA sequences changes dramatically during the early stages of development, extending the previous observation that linear DNA molecules rapidly ligate and concatemerize after injection into unfertilized eggs²⁴. This same ligation phenomenon seems to be occurring after the injection of the 6-kb linear sea urchin histone DNA into fertilized eggs.

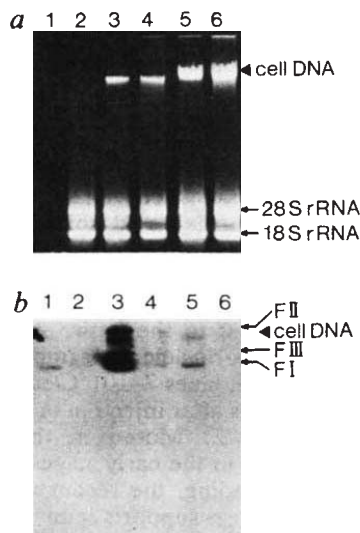


Fig. 2 Analysis by DNA blotting of the persistence and physical state of injected DNA sequences in developing embryos of *X. laevis* after the injection of fertilized eggs with supercoiled circles of sea urchin histone plus plasmid DNA. Circular, supercoiled pBR322 plasmid DNA containing two tandem copies of the h22 6-kb *Hind*III fragment was purified by CsCl gradient centrifugation and used to inject fertilized eggs. The injection procedure and subsequent analysis of embryonic nucleic acids were as described for injection of linear DNA molecules (Fig. 1). Again, a is the ethidium-stained gel and b the corresponding autoradiograph. As in Fig. 1, lane 1 is a control sample representing the amount of DNA injected per egg (250 pg). Lanes 2–6 represent one embryo equivalent of nucleic acid extracted at stage 1 (egg), stage 8 (blastula), stages 10–12 (gastrula), stage 22 (early tail-bud) and stage 40 (swimming tadpole), respectively.

Thus, by late blastula, most of the hybridizable sequences co-migrate with high-molecular-weight cell DNA rather than as 6-kb linear DNA molecules (Fig. 1, lane 3). Together, these results suggest that after injection into fertilized eggs, linear DNA molecules ligate and then replicate in the developing embryos as high-molecular-weight concatemers.

However, supercoiled, circular (FI) DNA injected into eggs is processed differently. Previous work has shown that when SV40 FI DNA is injected into oocyte nuclei, the FI DNA is first relaxed but within 10 h re-acquires a supercoiled structure²⁵. Figure 2, lane 2, illustrates that immediately following injection of FI DNA into fertilized eggs at least half of the injected FI DNA is relaxed. By the blastula stage, there has been a large increase in the total amount of injected DNA sequences per embryo, and most appear as FI DNA (Fig. 2, lane 3). Thus, relaxed DNA seems to be converted back to FI DNA and replicates as such. After injection of either linear or circular sea urchin DNA, at least some of the injected DNA sequences persist as DNA co-migrating with high-molecular-weight cell DNA (Fig. 1, lanes 5, 6; Fig. 2, lane 5). Although there is no direct evidence that any of the injected DNA sequences have become integrated into the *Xenopus* genome, this notion is supported, at least for a minor fraction of the injected DNA sequences, by the persistence of some of the sequences to later stages in the high-molecular-weight range.

Having determined that the microinjected sea urchin histone genes persist in the *Xenopus* embryos, at least during early development, the next question was whether they were also faithfully transcribed, as judged by S₁ mapping of mRNA termini²⁶. As the injected gene copy number is highest at about the late blastula stage, the initial experiments examined RNA from this stage. S₁ mapping experiments were performed using a multiple probe capable of detecting simultaneously all five sea urchin histone mRNA 5' termini. As shown in Fig. 3, lanes 2–4, three bands co-migrating with the 5'-terminal sequences of the authentic H2B, H2A and H3 mRNAs are clearly visible. There is a faint band corresponding to the 5'-terminal sequence of H1 mRNA, but no band corresponding to the protected 5'-terminal sequence of H4 mRNA was detected. Controls with RNA from uninjected *Xenopus* embryos show no bands of protected sequences (Fig. 3, lane 5). This pattern of S₁ protection is

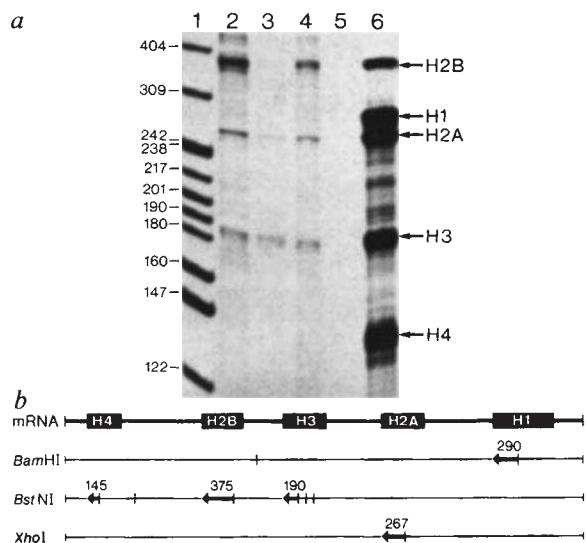


Fig. 3 Analysis by S_1 mapping of 5-14S RNA from *Xenopus* blastulae injected as eggs with sea urchin histone DNA. Artificially fertilized *Xenopus* eggs were injected with sea urchin histone DNA in either the linear or circular form as described in Figs 1 and 2 legends. Sucrose gradient-purified 5-14S RNA from the equivalent of 3.5 embryos (stage 9, late blastulae) was hybridized to a ^{32}P -5'-end-labelled probe of triple-digested (*Bam*HI-*Bst*NI-*Xho*I) 6-kb h22 DNA. As illustrated in panel b, this multiple probe is capable of protecting the 5' termini of all five sea urchin histone mRNAs from S_1 digestion⁷. S_1 mapping was performed as previously described^{7,26,34}. Lane 1 is a size marker of ^{32}P -5'-end-labelled *Hpa*II fragments of pBR322. Lanes 2-4 are RNA samples from injected embryos; lanes 2 and 4 were injected with linear DNA molecules, lane 3 with the circular form. Lane 5 is RNA prepared in parallel from uninjected embryos. Lane 6 is 5 μg of total RNA from *P. miliaris* embryos at the 128-cell stage of development.

identical to that previously established for transcription in the oocyte⁷, suggesting that the same factors are governing the production and accumulation of the mRNAs and that no developmental change has occurred. Interestingly, both the injection of circular and linear DNA templates gave this pattern although, as discussed above, the actual templates in the blastulae were probably either circles or high-molecular-weight concatemers; moreover, the circles contained DNA sequences from the plasmid vector pBR322. These results suggest that the transcriptional pattern is a fundamental reflection of the relative efficiency of the individual sea urchin histone promoter sequences within the *Xenopus* transcriptional machinery.

RNA from injected blastulae was also analysed for transcripts with the correct 3' termini. The 3'-terminal sequences of the *Psammechinus miliaris* H2B, H3, H2A and H1 histone mRNAs can be detected using an S_1 mapping multiple probe of ^{32}P -3'-end-labelled *Hpa*II-*Xho*I restriction fragments of h22 (ref. 7). Bands of protected fragments co-migrating with the protected 3'-terminal sequences of authentic H2B and H2A mRNAs were visible (not shown), but the correct 3'-terminal sequences of the H3 and H1 mRNAs were not detected. With either the 5'- or 3'-multiple probe, RNA from the injected embryos gave numerous faint bands of unknown protected sequences, indicating that although at least some of the correct 5' and 3' termini are synthesized, there is also some incorrect transcription.

RNA samples from progressive stages of development (from immediately following injection of the fertilized egg to the swimming tadpole stage) were also examined. The transcriptional expression pattern was found to be quite similar at all stages, with the overall level of sea urchin histone gene transcription apparently governed only by the abundance of the injected gene sequences rather than by the developmental stage of the *Xenopus* embryo. This result is not unexpected because genes from a heterologous source would probably not be expected to be brought under *Xenopus* developmental control. For example, adult rabbit β -globin gene was also found to be expressed in *Xenopus* embryos using the same microinjection protocol²⁷. Also, as has recently been shown for the 5S rRNA

genes²⁸, it is questionable whether naked DNA, even in a homologous system, respects developmental signals. Nevertheless, the approach described here paves the way for an assay capable of investigating what the units responding to developmental signals actually are and whether, for example, integration of genes into the correct chromosomal location is necessary before such signals are effective.

I thank S. Rusconi and C. C. Hentschel for assistance in egg injection and S_1 mapping, C. C. Hentschel, W. Schaffner and M. L. Birnstiel for critical reading of the manuscript, and S. Oberholzer and F. Ochsenbein for help in preparing the manuscript. This work was supported by a Swiss Nationalfonds fellowship to M.M.B. under the sponsorship of M. L. Birnstiel and W. Schaffner.

Received 7 January; accepted 14 April 1981.

- Kressmann, A., Clarkson, S. G., Telford, J. L. & Birnstiel, M. L. *Cold Spring Harb. Symp. quant. Biol.* **42**, 1077 (1977).
- Kressmann, A., Clarkson, S. G., Pirrotta, V. & Birnstiel, M. L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1176 (1978).
- Cortese, R., Melton, D., Tranquilla, T. & Smith, J. O. *Nucleic Acids Res.* **5**, 4593 (1978).
- De Robertis, E. M. & Olson, M. V. *Nature* **278**, 137 (1979).
- Brown, D. D. & Gurdon, J. B. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2849 (1978).
- Probst, E., Kressmann, A. & Birnstiel, M. L. *J. molec. Biol.* **135**, 709 (1979).
- Hentschel, C., Probst, E. & Birnstiel, M. L. *Nature* **288**, 100 (1980).
- Trendelenburg, M. F. & Gurdon, J. B. *Nature* **276**, 292 (1978).
- Trendelenburg, M. F., Mathis, D. & Oudet, P. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5984 (1980).
- Etkin, L. D. & Maxson, R. E. *Dev. Biol.* **75**, 13 (1980).
- Wickens, M. P., Woo, S., O'Malley, B. W. & Gurdon, J. B. *Nature* **285**, 628 (1980).
- DeRobertis, E. M. & Mertz, J. E. *Cell* **12**, 175 (1977).
- Rungger, D. & Türlér, H. *Proc. natn. Acad. Sci. U.S.A.* **75**, 6073 (1978).
- Kressmann, A., Hofstetter, H., Di Capua, E., Grosschedl, R. & Birnstiel, M. L. *Nucleic Acids Res.* **7**, 1749 (1979).
- Hofstetter, H., Kressmann, A. & Birnstiel, M. L. *Cell* **24**, 573 (1981).
- DeFranco, D., Schmit, O. & Söll, D. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3365 (1980).
- Sakonju, S., Bogenhagen, D. F. & Brown, D. D. *Cell* **19**, 13 (1980).
- Bogenhagen, D. F., Sakonju, S. & Brown, D. D. *Cell* **19**, 27 (1980).
- Grosschedl, R. & Birnstiel, M. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1432 (1980).
- Grosschedl, R. & Birnstiel, M. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 7102 (1980).
- Clarkson, S. G., Smith, H. O., Schaffner, W., Gross, K. W. & Birnstiel, M. L. *Nucleic Acids Res.* **3**, 2617 (1976).
- Gross, K., Schaffner, W., Telford, J. & Birnstiel, M. L. *Cell* **8**, 479 (1976).
- Harland, R. M. & Laskey, R. A. *Cell* **21**, 761 (1980).
- Clerc, R. thesis, Univ. Zürich (1980).
- Wyllie, A. H., Laskey, R. A., Finch, J. & Gurdon, J. B. *Dev. Biol.* **64**, 178 (1978).
- Hentschel, C., Irmlinger, J. C., Bucher, P. & Birnstiel, M. L. *Nature* **285**, 147 (1980).
- Rusconi, S. & Schaffner, W. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Korn, L. J. & Gurdon, J. B. *Nature* **289**, 461 (1981).
- Billeit, F. S. & Wild, A. E. *Practical Studies of Animal Development*, 119-127 (Chapman & Hall, London, 1975).
- Gurdon, J. B. & Brown, D. D. in *The Molecular Biology of the Mammalian Gene Apparatus* (ed. Tso, P.) 111-123 (Elsevier, Amsterdam, 1977).
- Nieuwkoop, P. D. & Farber, J. *Normal Table of Xenopus laevis* (Daudin) (North-Holland, Amsterdam, 1967).
- Wahl, G. M., Stern, M. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3683 (1979).
- Southern, E. M. *J. molec. Biol.* **98**, 503 (1975).
- Berk, A. J. & Sharp, P. A. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1274 (1978).

Preferential adhesion of tectal membranes to anterior embryonic chick retina neurites

Willi Halfter, Michael Claviez & Uli Schwarz

Max-Planck-Institut für Virusforschung, Abt. Biochemie, D-7400 Tübingen, FRG

Recognition between cells during embryogenesis is essential for the formation of precisely patterned neuronal networks such as the projection of axons from the retina on to the tectum in the avian optical system. A gradient of adhesion between retinal and tectal cells has been detected, with cells from the dorsal area of the neural retina adhering preferentially to cells of the ventral half of the visual tectum and vice versa (for review see ref. 1). This has supported the idea that topological relationships between cells are established through markers on the cell surface². Such findings are based on adhesion between cell bodies, whereas it is only the axon tip of the retina ganglion cell which makes contact with and presumably recognizes tectal cells. Here, we have used a system in which outgrowing neurites from the retina were exposed to tectal cell surfaces *in vitro* and found a striking antero-posterior polarity in the chick retina.

Fig. 1 Adhesion of tectal membranes to neurites from anterior and posterior retina explants. A retina from a 6-day White Leghorn chick embryo was isolated. Squares ($150 \times 150 \mu\text{m}$) from the anterior and posterior region were explanted on a collagen-coated dish (Falcon 30001 F) and incubated at 37°C in $4\% \text{CO}_2$ for 2 days (2 ml of Eagle's minimal essential medium with $2.4\% \text{NaHCO}_3$ and 5 mM HEPES , 10% fetal calf serum, 2% chick serum, 1% beef embryo extract, 2 mM glutamine , penicillin and streptomycin; all from Gibco). Finally, $30 \mu\text{l}$ of a suspension of tectal membranes (see below; $150\text{--}200 \mu\text{g protein}^{10,11}$) were added and incubation continued for another 2 h with gentle shaking at 30-min intervals. The medium was sucked off, the dish washed six times with prewarmed medium and the cultures were stained with Sudan black by a modification of the method of Wood¹² (after fixation with 2.5% glutaraldehyde in phosphate-buffered saline, impregnation in $0.19\% \text{OsO}_4$ and $0.5\% \text{K}_2\text{Cr}_2\text{O}_7$). Coating of the dish with collagen: native collagen was prepared essentially according to Elsdale and Bard¹³ (final concentration $350 \mu\text{g ml}^{-1}$). At the onset of gelling, the dish was turned into a vertical position leaving a thin layer of collagen. The membranes were prepared by a modification of the procedure of Brunette and Till¹⁴. Tecta from 20 9-day-old chick embryos were homogenized in 4 ml of 10 mM Tris , 1.5 mM CaCl_2 and 1 mM spermidine ($\text{pH } 7.4$) in a Dounce homogenizer. After centrifugation (10 min at $1,000g$) membranes were enriched in a biphasic system: a solution of dextran ($13.1g$ dextran T-500, Pharmacia, in $67.8 \text{ ml H}_2\text{O}$ bidest.) and a solution of polyethylene glycol 6000 (Carbowax, $8.95g$, in $67.8 \text{ ml H}_2\text{O}$ bidest.) were heated for 20 min at 80°C , cooled and added to a solution of NaCl (15 mg NaCl in $113.4 \text{ ml } 0.22 \text{ M Na-phosphate buffer, pH } 7.0$). After shaking and standing for 2 days at 4°C , two phases had formed. The upper phase (polyethylene glycol) and lower (dextran) phases were separated and sterilized by membrane filtration. The membranes were homogenized in 5 ml of the polyethylene glycol solution and 5 ml of the dextran phase was added. The two phases were mixed and centrifuged in a swing-out rotor for 10 min at $1,000g$. Membranes from the interphase were resuspended in 4 ml polyethylene glycol. After addition of 4 ml dextran phase and mixing, separation was repeated by centrifugation for 10 min at $23,500g$ in a Sorvall HB4 rotor. The membranes were again collected at the interphase and washed twice in culture medium. Based on the specific activity of marker enzymes³ (Mg^{2+} -ATPase, $\text{Na}^+ - \text{K}^+$ -ATPase and alkaline phosphatase), the membranes were enriched by a factor of four over the homogenate. The recovery of alkaline phosphatase in the membrane was 30% of the enzyme activity found in unfractionated homogenate. *a*, Explants from the anterior half of the retina (right) and from the posterior half of the retina (left) grown on the same culture dish; *b*, *c*, explants at higher magnification.

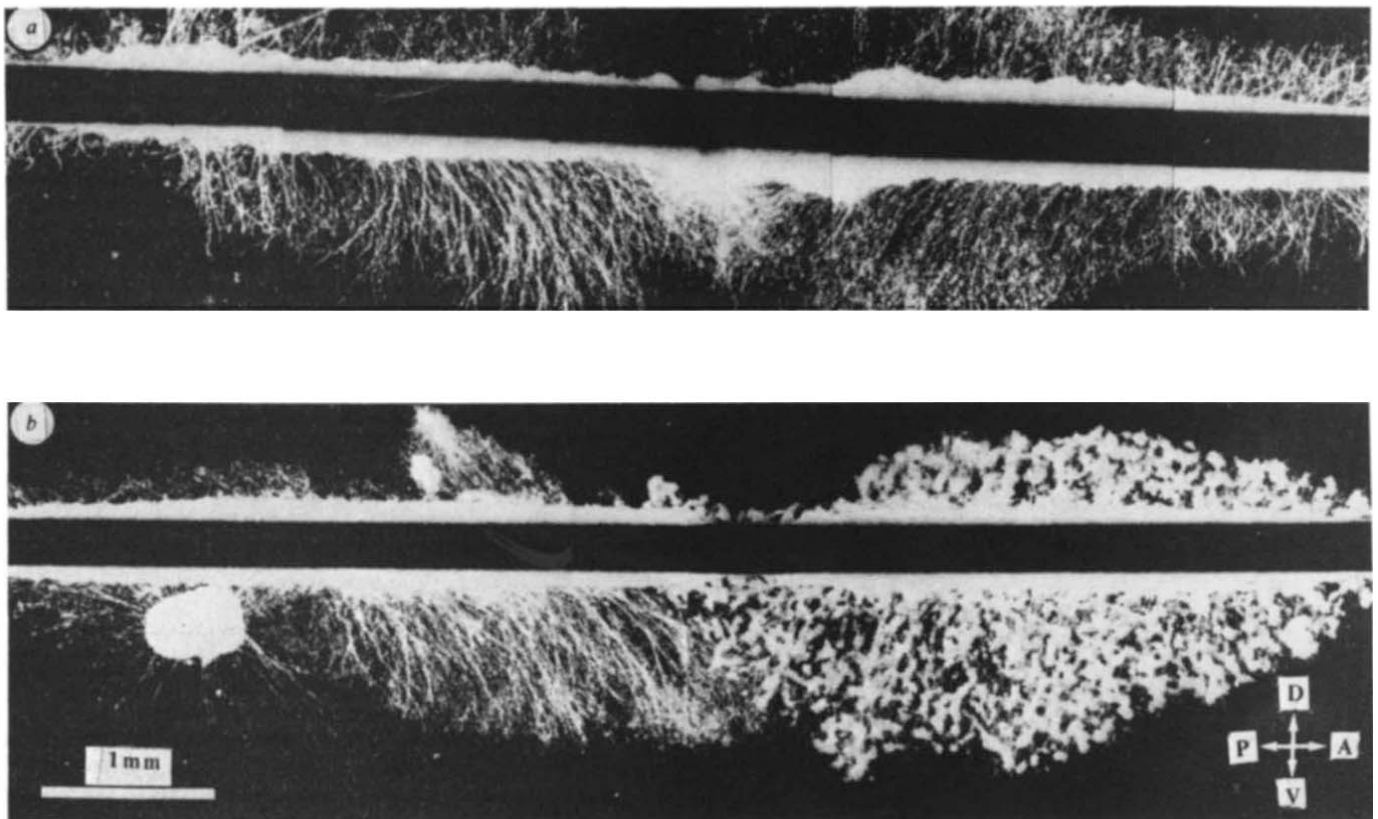
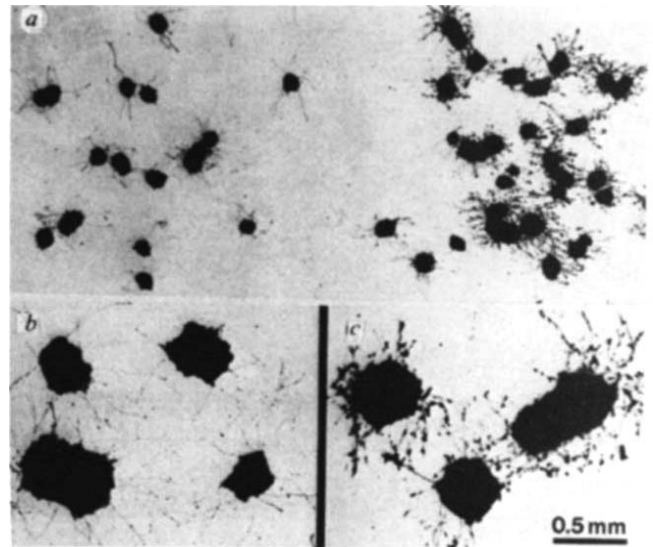


Fig. 2 Adhesion of tectal membranes to antero-posterior strips from the dorsal retina. A retina from a 6-day-old White Leghorn chick embryo was isolated intact and spread on a membrane filter (SM 13006, black; Sartorius); the ganglion cell layer was oriented upwards. The retina and membrane filter were cut into 0.3-mm strips in the antero-posterior direction, inverted on to collagen-coated dishes, and cultured as before, the ganglion cell layer now facing the collagen (Fig. 1), with the exception that a thicker collagen layer was used (0.7 ml collagen solution was allowed to form a gel in Petriperm dishes; Heraeus). After incubation for 30 h , a suspension of tectal membranes (see Fig. 1) was added. After incubation for 2 h the unbound membranes were washed off (see Fig. 1) and the specimen observed directly by dark-field illumination. *a* Is a control without membranes added, showing the oriented outgrowth of fibres towards the original choroid fissure which is oriented vertically through the centre of the strip; *b* shows the specific adhesion of tectal membranes to the neurites sprouting from the anterior half of the retina. The original orientation of the retina in the embryo is indicated by the windrose (D, dorsal; A, anterior; V, ventral; P, posterior).

Retina from 6-day chick embryos, when explanted and incubated on collagen, shows vigorous fibre growth. Using the choroid fissure as a topographical marker, small pieces of retina from defined regions were explanted. After 2 days in culture, a suspension of tectal cells, isolated by mechanical dissociation, was added and the incubation continued for a further 2 h with gentle shaking at 30-min intervals. Finally, the culture was washed and observed by dark-field illumination. In these experiments, preferential adhesion of tectal cells to neurites growing out from anterior retinal explants was observed. However, clarity of the results was impaired by the fact that the cells adhered to collagen and also to each other (data not shown). A much sharper distinction was obtained using membrane preparations from tectal cells, instead of whole cells. A striking preferential adhesion of tectal membranes to neurites from explants of anterior retina, as opposed to neurites growing out from posterior sections, was observed (Fig. 1). Identical results were obtained with membranes prepared by the elaborate procedure of Merrell and Glaser³.

The specific adhesion of tectal membranes to anterior neurites could reflect a continuous gradient or a discontinuous step of adhesiveness in the antero-posterior dimension of the retina. To decide on this alternative the retina was spread on a membrane filter and cut into narrow strips with a tissue chopper. The retinal strips were transferred on to collagen with the filter as a mechanical support. Using the choroid fissure for orientation, strips were cut in antero-posterior and in dorso-ventral directions. Such explants produced neurites *in vitro*. In agreement with results reported by Bonhoeffer and Huf⁴, their orientation is similar to that of axons *in vivo*⁵, that is, neurites grew centripetally towards the original position of the choroid fissure (Fig. 2a). Antero-posterior strips were explanted and incubated for 30 h and the adhesion of tectal membranes to the neurites was then tested as before. Microscopic examination showed very clearly two distinct areas with a sharp boundary; neurites from the anterior part of the retina were heavily labelled with

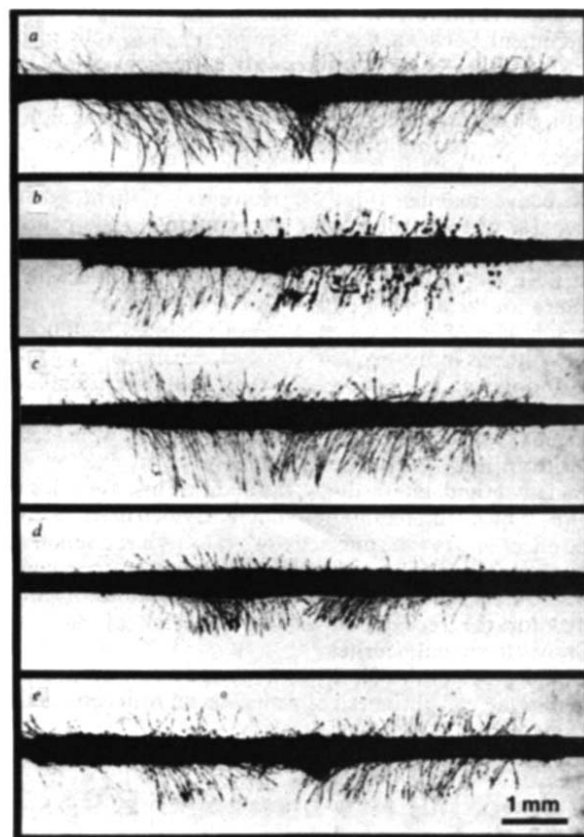


Fig. 4 Adhesion of membranes of different origin to neurites sprouting from antero-posterior sections through chick retina. The retina sections were prepared and incubated as described. After incubation for 30 h, membrane preparations from different parts of the central nervous system were prepared and the samples processed as described in Fig. 1 legend. *a*, Untreated control; the remaining preparations were treated with: *b*, membranes from optic tectum; *c*, membranes from brain prepared after removal of the optic tectum; *d*, membranes from retina and *e*, from fore-brain. The membranes had all been prepared from 9-day-old White Leghorn chick embryos.

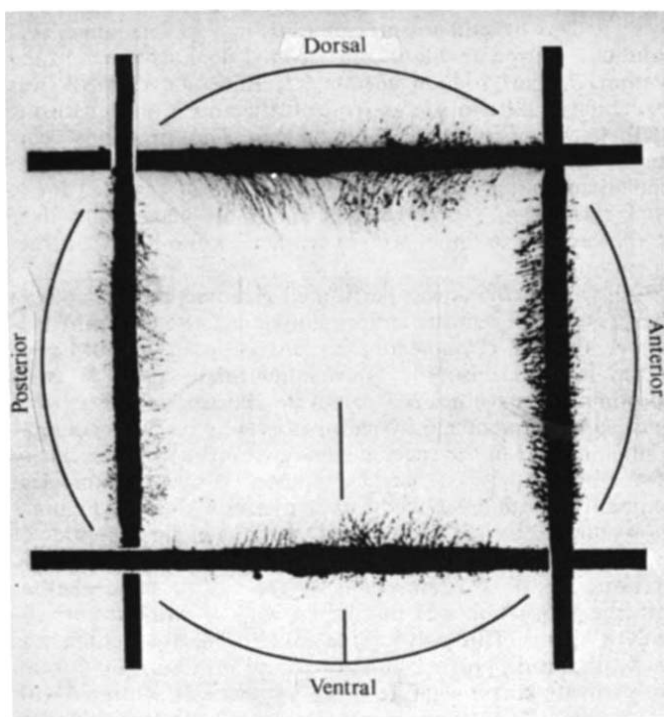


Fig. 3 Composite of four photographs taken from strips of the retina cut as indicated, explanted, incubated and treated with tectal membranes as described (Fig. 2). The antero-posterior sections from the dorsal and ventral parts of the retina came from one retina; the other two sections from another retina of a 6-day-old chick embryo. The samples were stained with Sudan black (Fig. 1) before photography. Over 200 experiments yielded identical results. The vertical line indicates the position of the choroid fissure; the strips are 0.3 mm wide.

membranes, in contrast to the posterior fibres (Fig. 2b). The neurites from dorso-ventral strips through the anterior part of the retina were covered with large amounts of membrane material whereas on dorso-ventral strips through the posterior part of the retina much less membrane material was bound. Only by scanning electron microscopy could membrane vesicles be detected on posterior neurites. A composite of photographs taken from four sections through the anterior, ventral, posterior and dorsal regions of the retina indicates the distinctive adhesion of tectal membranes to neurites originating from the anterior half of the retina, independently of the dorso-ventral coordinate (Fig. 3). All results indicate that neurites from the anterior and posterior part of the retina differ strikingly in binding tectal membranes. A similar antero-posterior polarity in retina can also be seen after a modification of the test system⁴ in which neurites growing out on cell monolayers can choose between retina and tectum cells; anterior neurites prefer tectum cells, posterior neurites do not (F. Bonhoeffer, personal communication).

The sprouting of neurites from retina explants reflects the time course of axon outgrowth *in vivo*⁵, which occurs between days 5 and 8. All stages within this range showed the specific binding of tectal membranes to anterior neurites. The age of the tectum used for membrane preparation was not relevant for adhesion; membranes from 6–13-day-old embryos all showed adhesion to anterior neurites. Retinal axons do not reach the tectum until day 6 of development^{6–8}, indicating that the neurites do not recognize retina-derived components on the tectum. It remains to be studied whether the neurites *in vitro* are true axons which originate mainly from ganglion cells, and whether they grow out *de novo* or are due to

regeneration. However, fibre orientation, time of outgrowth and the agreement between the number of ganglion cells in our explants and the number of fibres (M.C., in preparation), are consistent with a ganglion cell origin.

The origin of the membranes is crucial for the recognition by neurites. In contrast to the membranes from the optic tectum, membranes from fore-brain were not bound to anterior neurites in a selective manner (Fig. 4). However, a slight general adhesiveness of these membrane preparations to the neurites was observed (Fig. 4c-e). Membranes from non-neural tissue such as liver also showed some adhesion to all neurites with no preference for the anterior part.

The adhesion of membranes was not inhibited by blocking protein synthesis in the explant with cycloheximide ($5 \mu\text{g ml}^{-1}$) before and during incubation with the membranes. Similarly, the addition of concanavalin A ($2.5 \mu\text{g ml}^{-1}$) and colchicine (12.5 ng ml^{-1}) showed no effect at concentrations which interfered with further neurite elongation. However, fixation with glutaraldehyde and, interestingly, the addition of cytochalasin B ($5 \mu\text{g ml}^{-1}$) blocked membrane-binding. Cytochalasin B has a specific effect on growth cone activity⁹, causing a retraction and wilting of the microspikes, by which the axonal environment is explored. Intact function of growth cones, therefore, might be essential for the recognition and/or adhesion of the tectal membranes to retinal neurites.

The antero-posterior polarity as we found it *in vitro* might be involved in the establishment of retino-tectal connections dur-

ing *in vivo* development; this assumption, however, requires further study. In any case, the findings clearly show that the retina consists of subareas distinguishable by their interactions with tectal membranes; they provide a relatively simple assay for further studies of this interaction. For example, we can test whether the antero-posterior polarity in the retina has a counterpart in the tectum. Furthermore, a biochemical characterization of the components involved in the interaction of the neurites with the tectal membranes should be possible.

We thank Dr Dütting for advice on tissue culture, F. Bonhoeffer, A. Gierer, D. Dütting for helpful discussions and D. Newgreen for suggestions in preparing the manuscript.

Received 4 December 1980; accepted 24 April 1980.

1. Gottlieb, D. I. & Glaser, L. A. *Rev. Neurosci.* **3**, 303-318 (1980).
2. Sperry, R. W. *Proc. natn. Acad. Sci. U.S.A.* **50**, 703-710 (1963).
3. Merrell, R. & Glaser, L. *Proc. natn. Acad. Sci. U.S.A.* **70**, 2794-2798.
4. Bonhoeffer, F. & Huf, J. *Nature*, **288**, 162-164 (1980).
5. Goldberg, S. & Coulombre, A. J. *Comp. Neurol.* **146**, 507-517.
6. Goldberg, S. *Dev. Biol.* **36**, 24-43 (1974).
7. Crossland, W. J., Cowan, W. M. & Rogers, L. A. *Brain Res.* **91**, 1-23 (1975).
8. Rager, G. & von Oeynhausen, B. *Expl Brain Res.* **35**, 213-227 (1979).
9. Yamada, K. M., Spooner, B. S. & Wessels, N. K. *J. Cell Biol.* **49**, 614-635 (1971).
10. Kalb, V. F. & Bernlohr, R. W. *Analyt. Biochem.* **82**, 362-371 (1977).
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. *J. biol. Chem.* **193**, 265-275 (1951).
12. Wood, P. M. *Brain Res.* **115**, 361-375 (1976).
13. Elsdale, T. & Bard, J. *J. Cell Biol.* **54**, 626-637 (1972).
14. Brunette, D. M. & Till, J. E. *J. Membrane Biol.* **5**, 215-224 (1971).

One gene, but two messenger RNAs encode liver L and red cell L' pyruvate kinase subunits

Joelle Marie, Marie-Pierre Simon,
Jean-Claude Dreyfus & Axel Kahn

Institut de Pathologie Moléculaire, INSERM U 129,
Chu Cochin 75674 Paris Cedex 14, France

Pyruvate kinase subunits of red cells and liver differ in their molecular weights. Peptide mapping of the rat enzymes has shown that this difference is due to a single exon peptide present in the red cell enzyme¹⁻³. There is strong genetic evidence in man that both enzymes are encoded by the same structural gene (refs 4-8 and G. E. J. Staal *et al.*, personal communication). Here we describe *in vitro* protein synthesis experiments using RNA extracted from rat red cells and liver which demonstrate that the difference is reflected in tissue-specific mRNAs. Thus the difference is not due to post-translational processing and presumably involves either gene rearrangement or differential processing of a common nuclear RNA precursor.

In our laboratory, we have demonstrated that pyruvate kinase is synthesized in red cells as L' subunits (M_r 63,000) and in liver as L subunits (M_r 60,000). *In vitro*, the L' tetramers can be converted by mild tryptic attack into a form with molecular weight and properties similar to liver L₄ pyruvate kinase⁹⁻¹². As both trypsin-treated red cell enzyme and liver pyruvate kinase exhibit improved regulatory properties with respect to native L' (refs 9, 12), it was assumed that, in liver, L-type pyruvate kinase undergoes a proteolytic processing. To test this model we have characterized pyruvate kinase neosynthesized in a rabbit reticulocyte lysate system under the direction of human liver RNA, rat liver RNA or rat reticulocyte RNA. If the L and L' subunits differ due to a proteolytic processing, then reticulocyte and liver RNAs should direct the synthesis of identical subunits with an identical molecular weight to L.

Anti-human red cell pyruvate kinase antiserum was raised in rabbits and specific antibodies purified by chromatography on a pyruvate kinase-linked agarose bead column¹³. The purified

antibodies were then coupled to agarose beads. By exploiting the cross-reactivity between human and rat enzymes, it was possible, using this immunoabsorbent, to purify pyruvate kinase from human liver, rat liver and rat red cells in only one step (Fig. 1). Rat L' and L subunits were slightly heavier than their human counterparts (M_r s 64,000 and 61,000 respectively). Reticulocytosis was induced in rats by treatment with acetylphenylhydrazine¹⁴. Total cellular high-molecular-weight RNA was purified by ethanol precipitation in 7 M guanidine/HCl solution followed by chloroform/isoamyl alcohol extraction and washing in 3 M sodium acetate^{13,15}. Reticulocyte RNA was fractionated by two successive centrifugations on denaturing methylmercury/formamide gradients. The fractions corresponding to sedimentation coefficients 18S-~24S were pooled and, after ethanol precipitation, RNA was used for *in vitro* translation. Total cellular high-molecular-weight liver RNA was used to direct protein synthesis without further fractionation.

Cell-free synthesis was performed, in some experiments, in the presence of various antiproteolytic agents: 0.01 mM leupeptin, 0.1 mM chymostatin, 0.1 mM antipain, 0.1 mM pepstatin, 0.1 mM diisopropylphosphorofluoridate and 1% (v/v) aprotinin. Neosynthesized pyruvate kinase subunits were purified by immunoaffinity chromatography on microcolumns containing 5 μl of the specific immunoabsorbents¹³. Specificity of the neosynthesized bands was checked by immunological competition with unlabelled L-type pyruvate kinase¹³. Figure 2 shows that rat liver RNA directed synthesis of a polypeptide of M_r 61,000—the same molecular weight as rat liver L-type pyruvate kinase. This molecular weight was the same whether cell-free translation was performed with or without antiproteolytic agents. This polypeptide could be firmly identified as neosynthesized pyruvate kinase because it did not bind to an antipyruvate kinase-agarose column previously saturated with excess unlabelled L-type enzyme but was specifically retained by a nonsaturated column (Fig. 3). The same results were obtained with human liver RNA (not shown).

Figure 4 shows that, by contrast, rat reticulocyte RNA directed synthesis of a 64,000- M_r polypeptide (thus similar to rat L') which immunologically competed with unlabelled L-type enzyme for binding to the antibody column. When a mixture of reticulocyte and liver RNAs was translated together, both the

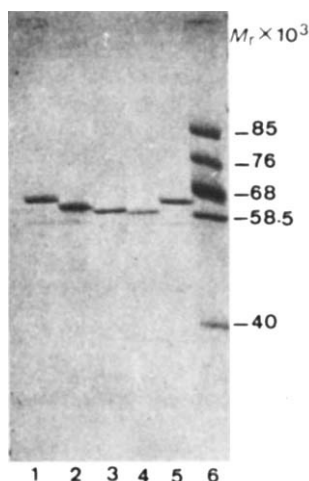


Fig. 1 Purification of red cell and liver pyruvate kinase from man and rat by immunoaffinity microchromatography. Cells or tissues were lysed in a 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 0.1 mM β -mercaptoethanol, 10 mM EDTA, 10 mM ϵ -aminocaproic acid, 2% (v/v) aprotinin, 1 mM diisopropylphosphofluoridate, 0.1 mM pepstatin, 0.1 mM chymostatin, 0.1 mM antipain, 0.01 mM leupeptin, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate (= lysis buffer). After elimination of the insoluble cell debris by centrifugation (at 36,000 g for 20 min), the extracts were applied to microcolumns made with Eppendorf blue tips and containing 10 μ l of immunoabsorbent (4 mg of specific anti-L-type pyruvate kinase immunoglobulins per ml of resin). About 3 IU of pyruvate kinase were bound per column. The columns were washed successively with 5 ml of lysis buffer, 5 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 1 M KCl and 5 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. They were eluted with 800 μ l of 0.1% (w/v) SDS in 10% (v/v) acetic acid. The eluates were lyophilized and the lyophilisates dissolved in 40 μ l of 0.0685 M Tris/HCl buffer (pH 6.8) containing 10% glycerol (v/v), 5% β -mercaptoethanol (v/v) and 0.01% bromophenol blue (w/v); dodecylsulphate concentration of these samples was therefore 2% (w/v), such that they could be directly applied, after dissociation (2 min in boiling water), to dodecylsulphate/polyacrylamide gradient gel electrophoresis according to Laemmli²⁷. Proteins were stained with Coomassie brilliant blue. 1, rat red cell; 2, rat liver; 3, human liver; 4, trypsin-treated human red cell enzyme⁹; 5, untreated human red cell enzyme; 6, protein markers: urease (M_r 85,000), transferrin (76,000), bovine serum albumin (68,000), glucose phosphate isomerase (58,500) and half urease (40,000). The faint band corresponding to a M_r of ~57,500 observed in the pyruvate kinase preparations corresponds to Lc, a partially proteolysed form¹¹.

61,000 and 64,000 bands could be detected (not shown). This result, and the fact that antiproteolytic agents did not modify the molecular weight of neosynthesized liver pyruvate kinase, exclude the possibility that neosynthesized chains are cleaved by proteases present in the reticulocyte lysate or by neosynthesized proteases. Clearly, therefore, in rat, L and L' pyruvate kinase subunits are synthesized under the direction of two different species of mRNA. Although it was not possible to study human reticulocyte RNA, the same conclusion can probably be drawn in man because human liver RNA directs synthesis of L, and not L' as predicted by the hypothesis of the 'proteolytic processing'.

As studies of inherited red cell pyruvate kinase deficiency indicate unambiguously that L and L' subunits are encoded by the same gene, our results strongly suggest that this gene is associated with two types of mRNA present in the different cells—erythroid and liver. Two basic mechanisms could explain this finding: a rearrangement of the L-type pyruvate kinase gene during erythroid or liver differentiation, or a differential processing of a common nuclear RNA precursor.

In lower eukaryotes, a gene rearrangement mechanism has been implicated, such as in the yeast mating system^{16,17} and modification of trypanosome surface antigens¹⁸.

In vertebrates, gene rearrangement has been described only for immunoglobulin genes during B-cell differentiation¹⁹⁻²¹. Differential processing of nuclear RNA has been described for immunoglobulin μ chains²²⁻²⁴, where both types of μ chain, and therefore both types of mRNA, are produced in the same cells, whereas in the case of pyruvate kinase, L' is the only form produced in the reticulocytes and L the only form in the liver. Differential splicing of nuclear RNA in salivary glands and liver^{25,26} has also been shown for mouse α -amylase; in this case the two species of mRNA encode the same protein. These results suggest that differentiation can be associated with changes of protein form that are not directly related to the usual phenomena of gene activation and inactivation, but rather to modifications of gene arrangement or of processing of nuclear RNA into cytoplasmic translatable mRNA. The study of pyruvate kinase genomic DNA in different cells should elucidate this problem, but requires a cDNA probe which may be difficult to obtain because of the very low amount of specific mRNA:

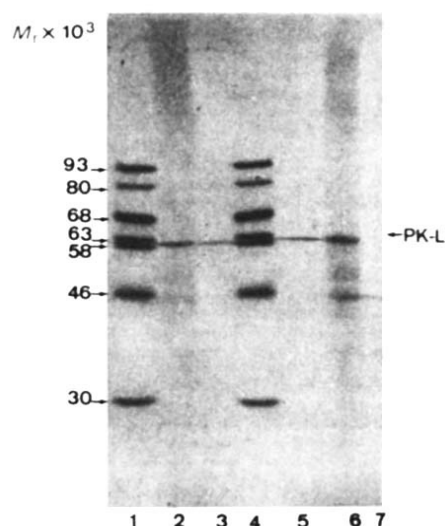


Fig. 2 Characterization of neosynthesized liver L-type pyruvate kinase subunits. Rabbit reticulocyte lysate was deprived of endogenous RNA by micrococcal nuclease treatment, according to ref. 28. Translation mixture (250 μ l) contained, in final concentration, 20 μ M essential amino acids except methionine, 0.5 mCi ³⁵S-methionine (specific activity 1,000 Ci mmol⁻¹), 1 mM ATP, 0.2 mM GTP, 0.3 mM spermidine, 1 mM dithiothreitol, 10 μ M haemin, 10 mM creatine phosphate, 60 μ g ml⁻¹ creatine phosphokinase, 20 mM HEPES buffer, pH 7.4, 120 mM K acetate, 1.20 mM Mg acetate and 40 μ g liver RNA. Translation was carried out for 1 h at 37 °C, then the reaction was diluted with 2 vol of the 'lysis buffer' described in Fig. 1 legend and centrifuged at 36,000 g for 30 min. The diluted translation mixture was applied to two combined microcolumns (inserted into each other), containing 10 μ l nonimmune γ -globulin-linked resin at the top and 5 μ l anti-pyruvate kinase resin at the bottom. After about six passages through this system, the microcolumns were separated, individually washed and eluted as described in Fig. 1 legend. Lanes 1 and 4, ¹⁴C-labelled protein markers: rabbit muscle phosphorylase (M_r 93,000), human muscle phosphofructokinase (M_r 80,000), bovine serum albumin (M_r 68,000), human L₄ red cell pyruvate kinase (M_r 63,000), human glucose phosphate isomerase (M_r 58,500), hen egg ovalbumin (M_r 46,000) and bovine carbonic anhydrase (M_r 30,000). Lanes 2 and 6, eluates from anti-pyruvate kinase columns, synthesis without addition of antiproteolytic agents. 3 and 5, eluates from anti-pyruvate kinase columns, synthesis in the presence of 0.01 mM leupeptin, 0.1 mM pepstatin, 0.1 mM chymostatin, 0.1 mM antipain, 0.1 mM diisopropylphosphofluoridate, 1% (v/v) aprotinin. 7, eluate from a nonimmune column. The 43,000- M_r band, observed in the eluates from nonimmune systems as well as from some specific immunoabsorbent columns, can be identified as contaminant actin¹³. Fluorography was performed as in refs 29 and 30.

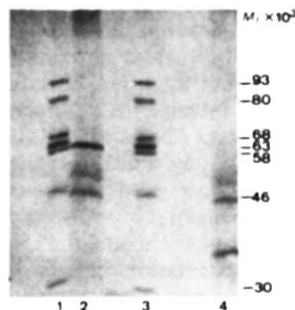


Fig. 3 Characterization of neosynthesized liver L-type pyruvate kinase by immunological competition. Cell-free translation was carried out as indicated in Fig. 2 legend, but the reaction was passed, successively, through saturated and nonsaturated anti-pyruvate kinase resins. The immunoabsorbent was saturated with 100 µg cold human L-type enzyme, then the resin was extensively washed before being used with the neosynthesized products. Lanes 1 and 3, ^{14}C -labelled protein markers (as in Fig. 2 legend). 2, eluate from a 5-µl nonsaturated anti-pyruvate kinase column. 4, eluate from 5-µl saturated anti-pyruvate kinase column. Detection of the radioactive bands by fluorography.

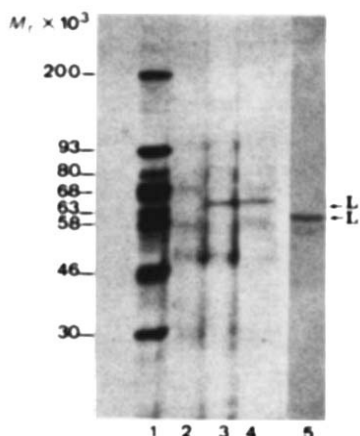


Fig. 4 Characterization of neosynthesized L' red cell pyruvate kinase by immunological competition. Comparison with neosynthesized L enzyme. Cell-free synthesis was directed by fractionated 18S–24S reticulocyte RNA (30 µg per 250 µl of translation medium) (lanes 2–4) or by rat liver RNA, as indicated in Fig. 2 legend (lane 5).

Fractionation of reticulocyte RNA was carried out by centrifugation in denaturing methylmercury formamide/sucrose gradient. 400 µg RNA were first dissolved in 20 µl 10 mM methylmercury, incubated at room temperature for 5 min, then mixed with 200 µl of 90% deionized formamide (v/v) in 10 mM Tris-HCl, pH 7.5, 10 mM iodoacetate, 5 mM EDTA and 0.5% lauroyl sarcosine. After a 15-min incubation at room temperature, this RNA solution was diluted with 250 µl of the Tris-HCl buffer described above, but without formamide, and applied to an 11-ml gradient sucrose: 10–25% (w/v) sucrose in 10 mM Tris-HCl, pH 7, containing 50% (v/v) deionized formamide, 10 mM iodoacetate, 5 mM EDTA and 0.5% (w/v) lauroyl sarcosine. Centrifugation was carried out in a SW 41 Beckman rotor, at 40,000 r.p.m. for 14 h. The 18S–24S RNA was pooled and precipitated by ethanol, and then applied to a second gradient. The 18S–24S fraction obtained from the second round was used for directing cell-free translation.

Lane 1, ^{14}C -labelled protein markers: myosin (M_r 200,000), phosphorylase, muscle phosphofructokinase, bovine serum albumin, L' human pyruvate kinase, glucose phosphate isomerase, ovalbumin, carbonic anhydrase and lactoglobulin A (M_r 18,500). Lane 2, synthesis directed by reticulocyte RNA, eluate from a 5-µl saturated anti-pyruvate kinase column. Lanes 3 and 4, synthesis directed by reticulocyte RNA; eluates from 5-µl nonsaturated anti-pyruvate kinase columns. Lane 5, synthesis directed by liver RNA, eluate from 5-µl nonsaturated anti-pyruvate kinase column. Detection by fluorography.

pyruvate kinase represents <0.05% of the proteins synthesized under the direction of liver RNA in cell-free conditions and this proportion is even less for reticulocyte RNA.

This work was supported by INSERM grant CRL 80-1028. We thank Dr Jacques Kruh and Fanny Schapira for helpful discussion and advice, and F. Carrouget for typing the manuscript.

Note added in proof: We recently succeeded in translating in a cell-free system both L and L' messenger RNA from fetal human liver, which confirms that in man as well as in rat L and L' pyruvate kinase subunits are encoded by two different species of messenger RNAs.

Received 22 December 1980; accepted 10 April 1981.

- Cleveland, D. W., Fisher, S. G., Kirschner, M. W. & Laemmli, U. K. *J. biol. Chem.* **252**, 1102–1106 (1977).
- Harada, K., Saheki, S., Wada, K. & Tanaka, T. *Biochim. biophys. Acta* **524**, 327–339 (1978).
- Saheki, S., Saheki, K. & Tanaka, T. *FEBS Lett.* **93**, 25–28 (1978).
- Bigley, R. H. & Koler, R. D. *Ann. hum. Genet.* **31**, 383–390 (1965).
- Kahn, A., Marie, J., Galand, C. & Boivin, P. *Scand. J. Haemat.* **16**, 250–257 (1976).
- Nakashima, K. *et al. Blood* **43**, 537–548 (1974).
- Nakashima, K. *et al. J. Lab. clin. Med.* **90**, 1012–1020 (1977).
- Shinohara, K. *et al. Am. J. hum. Genet.* **28**, 474–481 (1976).
- Kahn, A., Marie, J., Garreau, H. & Sprengers, E. D. *Biochim. biophys. Acta* **523**, 59–74 (1978).
- Marie, J., Garreau, H. & Kahn, A. *FEBS Lett.* **78**, 91–94 (1977).
- Marie, J. & Kahn, A. *Biochem. biophys. Res. Commun.* **91**, 123–129 (1979).
- Sprengers, E. D. & Staal, G. E. J. *Biochim. biophys. Acta* **570**, 259–270 (1979).
- Kahn, A., Cottreau, D., Daegelen, D. & Dreyfus, J. C. *Eur. J. Biochem.* **116**, 7–12 (1981).
- Hunt, T. & Jackson, R. J. in *Modern Trends in Human Leukaemia* (eds Neth. R., Gallo, R. C., Spiegelman, S. E. & Stohman, F.) 300–307 (Lehmanns, Munich, 1974).
- Cox, R. A. *Meth. Enzym.* **12**, 120–129 (1968).
- Hicks, J., Strathern, J. & Klar, A. J. S. *Nature* **282**, 478–482 (1979).
- Nasmyth, K. A. & Tatchell, K. *Cell* **19**, 753–764 (1980).
- Hoeijmakers, J. H. J., Frasch, A. C. C., Bernards, A., Borst, P. & Cross, G. A. M. *Nature* **284**, 78–80 (1980).
- Davis, M. M. *et al. Nature* **283**, 733–739 (1980).
- Early, P. W., Davis, M. M., Kaback, D. B., Davidson, N. & Hood, L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 857–861 (1979).
- Early, P., Huang, H., Davis, M., Calame, K. & Hood, L. *Cell* **19**, 981–992 (1980).
- Alt, F. W. *et al. Cell* **20**, 293–301 (1980).
- Early, P. *et al. Cell* **20**, 313–319 (1980).
- Singer, P. A., Singer, H. H. & Williamson, A. R. *Nature* **285**, 294–300 (1980).
- Hagenbüchle, O. *et al. Nature* **289**, 643–646 (1981).
- Young, R. A., Hagenbüchle, O. & Schibler, U. *Cell* **23**, 451–458 (1981).
- Laemmli, U. K. *Nature* **227**, 680–685 (1970).
- Pehlam, H. P. B. & Jackson, R. J. *Eur. J. Biochem.* **67**, 247–256 (1976).
- Bonner, W. M. & Laskey, A. R. *Eur. J. Biochem.* **46**, 83–88 (1974).
- Laskey, R. A. & Mills, A. D. *Eur. J. Biochem.* **56**, 335–341 (1975).

Nucleotide sequence of the haemagglutinin gene of a human influenza virus H1 subtype

Greg Winter*, Stan Fields* & George G. Brownlee†

*MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

†Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

Influenza remains a serious cause of disease in man and of death in the aged. Vaccination is only transiently effective in providing immunity¹, because the surface haemagglutinin molecule of the virus can evolve rapidly, allowing the virus to escape neutralization by immunizing antibody. Of the three influenza A subtypes² known to cause infection in man, the H1 subtype is of particular interest as it has proved to be the dominant human subtype this century and is now co-circulating with variants of the H3 subtype. Here we present the sequence of the haemagglutinin gene of an early H1 subtype (strain A/PR/8/34) as determined by recombinant DNA methods and dideoxy sequencing. From the deduced amino acid sequence of the haemagglutinin we define an antigenic site at amino acid residue 160. Comparison with haemagglutinin molecules^{3–9} shows that the H1 and H2 subtypes are more homologous to one another than to the other subtypes, which suggests that they have diverged only recently from one to another.

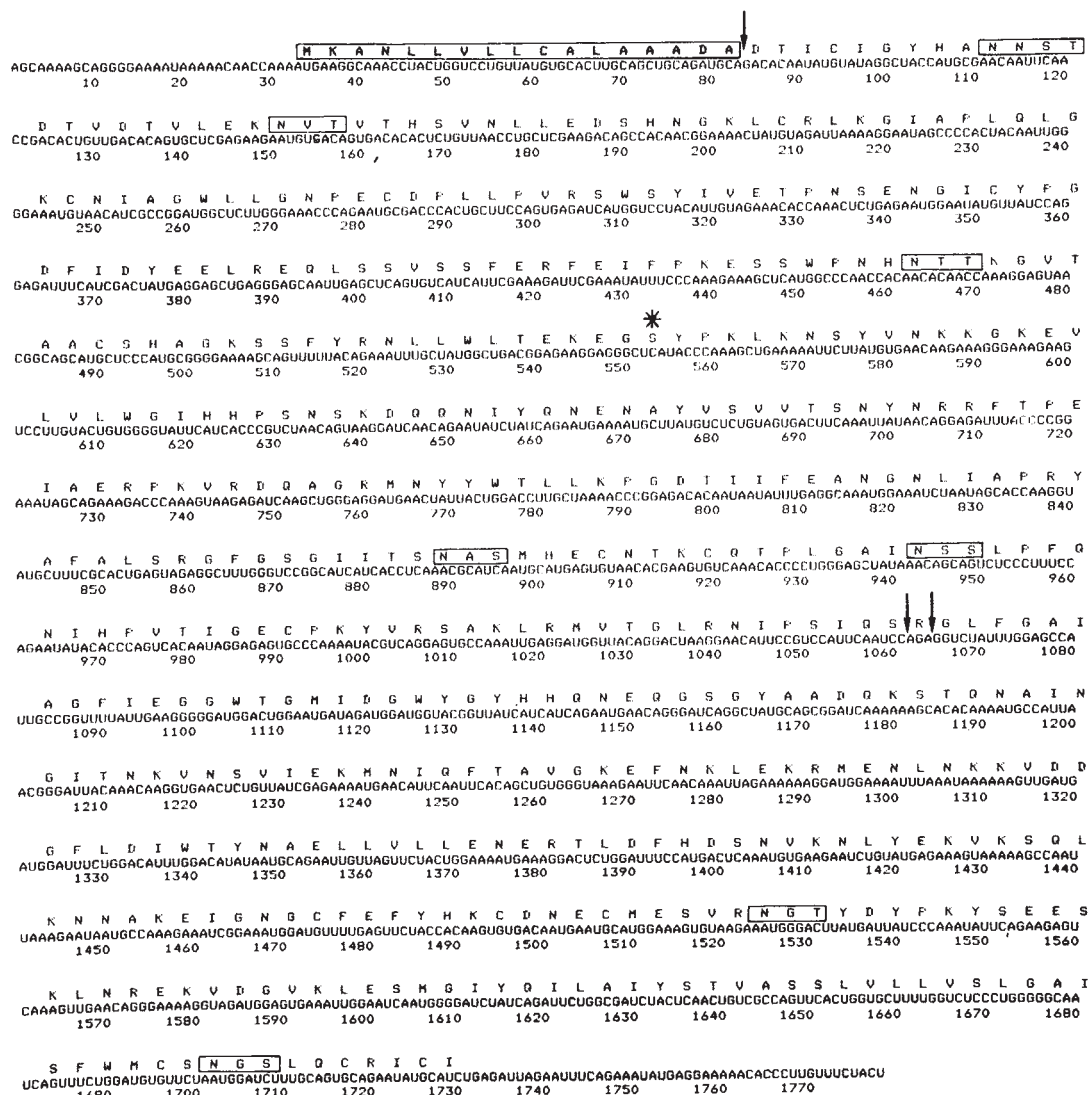
Influenza is a negative-stranded virus with eight RNA genes coding for 10 or more proteins, of which band 4 codes for the haemagglutinin gene. The main strategy for sequencing this gene was similar to that used in a parallel study of the neuraminidase gene¹⁰⁻¹². Briefly we prepared restriction fragments derived from double-stranded DNA, itself prepared by *in vitro* copying of the RNA gene 4 by reverse transcriptase. These restriction fragments were cloned into the bacteriophage M13 vector and sequenced by the Sanger method^{13,14}. By sequencing clones which were isolated at random and derived from several different restriction enzyme digests, we hoped to overlap these clones and deduce a unique sequence for the haemagglutinin gene. This objective was only 99% achieved; for the remaining 1% of the sequence we had to use an indirect RNA sequencing method¹⁵. We also used a direct RNA sequencing method to confirm the sequence of one end of the gene¹⁶.

Figure 1 shows the deduced nucleotide sequence, written in the mRNA sense. Its organization is similar to haemagglutinin molecules previously studied in other subtypes^{3-9,17}. We can identify a 17-residue hydrophobic signal peptide, an HA1 subunit (326 residues long) and an HA2 subunit (222 residues long). These, as in the other human subtypes, are separated by a single arginine residue (327) which is presumably recognized during processing by a trypsin-like enzyme. The HA2 subunit contains the characteristic conserved 14-amino acid long hydrophobic N-terminal sequence as well as a hydrophobic tail, believed to anchor the molecule to the phospholipid bilayer of the cell¹⁸.

Our studies provided no direct information on the secondary structure of the haemagglutinin, but we can deduce from the conservation of cysteine residues (Fig. 2) that the pattern of disulphide bonds is probably similar to that of the H2 and H3 subtypes, where most cysteines are involved in S-S bridges within a subunit, except for C(14) and C(466) (see Fig. 2) which interconnect the separate subunits¹⁹. There is no information on the three conserved cysteine residues close to the C terminus of the HA2 subunit. The haemagglutinin is a glycoprotein in which we observe seven potential N-X-S or N-X-T sequences (Fig. 1) where carbohydrate could be attached²⁰. There have been no direct studies of the location of the carbohydrate in A/PR/8/34, but studies of related strains (A/WSN/33)²¹ and other subtypes (A/Japan/57)⁴ suggest that the majority are glycosylated. Presumably the most N-terminal glycosylation site in the HA1 subunit (N-N-S-T—a sequence also conserved in the H2 subtype) is glycosylated at one but not both of the asparagine residues⁴.

Monoclonal antibodies have been previously used to select A/PR/8/34 variants which escape antibody neutralization because of their altered antigenicity²². A common variant contained a leucine residue replacing a serine residue in the parental tryptic peptide (SEPGY)K, although this peptide could not be further localized within the sequence. From the amino acid sequence predicted here (Figs 1, 2), we identify this variant as serine→leucine (160) caused by a C→U transition at base 553. Residue 160 (for numbering of amino acids, see Fig. 2

Fig. 1 The nucleotide and amino acid sequence of the haemagglutinin gene from A/PR/8/34 (Cambridge strain), written in the mRNA sense. The cleavage points at the N terminus of the HA1 and the HA1/HA2 junction are marked with an arrow. Small boxes mark potential glycosylation sites, the large box the signal peptide. The nucleotide sequence was established by the M13 refs 28-30) cloning-dideoxy sequencing procedure using a universal primer³² with *AluI*, *MboI*, *TaqI*, *HinfI*, *DdeI* (low-salt buffer) digests and *DdeI/EcoRI* double digests on double stranded DNA, and cloning into M13mp2, M13mp2(*Bam*) or M13mp7 (ref. 11). The asterisk marks the Ser→Leu change in an antigenic variant (see text). 76% of the sequence was cross-checked as it was present in M13 clones derived from both strands of DNA. The sequence of residues 1,398-1,412 was determined using an indirect RNA sequence method¹⁵. A restriction fragment corresponding to residues 1,222-1,273 was isolated from an *R_F* preparation of a suitable recombinant haemagglutinin clone and was used as a primer for dideoxy sequencing using reverse transcriptase and total virion RNA as template¹⁰. The 5' end of band 4 virion RNA was checked by direct RNA sequencing¹⁶. The computer programs of Staden³¹ were used to assist data handling and overlapping of fragments.



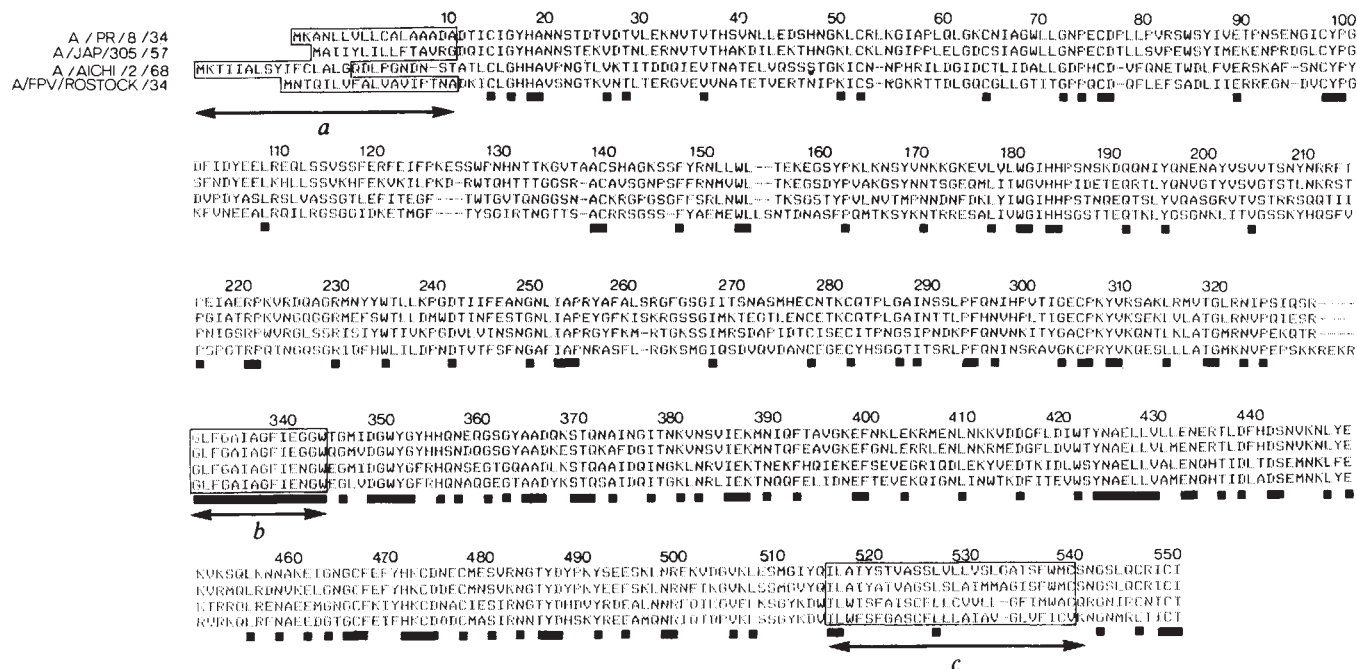


Fig. 2 The amino acid sequences of haemagglutinin from A/PR/8/34 (H1 subtype), A/Jap/305/57 (H2 subtype), A/Aichi/2/68 (H3 subtype) and A/FPV/Rostock/34 (H7 subtype) are listed with gaps inserted generally to maximize amino acid homology and particularly the alignment of cysteine, tryptophan and proline residues. The precise position of gaps is rather subjective but was finally decided after inspection of the three-dimensional structure of the H3 subtype²³. Gaps were then introduced into regions which, by analogy with the H3 subtype, might be expected to be in surface loops. The numbering system is based on consecutive amino acid residues (ignoring gaps) starting at the terminus of the H3 subtype (A/Aichi/2/68). Thus residue 160 of A/PR/8/34 is actually residue 157 from its N terminus, but is homologous to 160 of A/Aichi/2/68. The boxed areas *a*, *b* and *c* mark, respectively, the signal peptide region, the conserved N terminus of the HA1 subunit and the membrane attachment C-terminal tail.

legend) has not previously been implicated as an antigenic site in the better studied H3 subtype, despite the fact that the homologous residue threonine (160) seems to be on the surface of the haemagglutinin in the proposed site B in the recently available three-dimensional model of the H3 subtype²³. Perhaps the presence of the nearby carbohydrate side chain at residue 165 masks this region in the H3 subtypes. Further studies of other laboratory variants as well as field strains with altered antigenicity are required to define the antigenic regions of the H1 subtype in more detail.

Figure 2 shows a comparison of the amino acid sequence of the A/PR/8/34 haemagglutinin with representative strains of three other subtypes. The fact that only occasional gaps were required in one or other of the sequences to maintain alignment confirms the basic homology of all haemagglutinin subtypes. Furthermore, the conservation of cysteine and many proline residues predicts a common overall shape. Table 1 gives a quantitative measure of the extent of amino acid and nucleotide similarity between the different subtypes. This clearly shows that the H1 and H2 subtypes are more closely related to one another in both their HA1 and HA2 subunits than either is to the H3 or

H7 subtypes. The 58% amino acid conservation in the HA1 subunit of the H1 and H2 subtypes is far greater than the typical 33–36% similarity of the other possible comparisons. The fact that H1 and H2 subtypes are related is also apparent from the nucleotide sequence comparisons (Table 1).

The demonstrable homology of all haemagglutinin subtypes shows that they must have evolved from a common ancestral gene by a process of point mutation and the occasional deletion and addition. From the extent of antigenic drift in the human H3 subtypes, we know that change can occur quickly at a rate in the order of 1% amino acid change per year for the HA1 subunit (a value estimated from the extent of drift in the H3 series A/NT/60/68, A/Memphis/102/72 and A/Victoria/2/75)^{24,8}. Thus, in theory, if this rate of drift were maintained and were also applicable to the H1 subtype, the 42% difference in the amino acid sequence of the HA1 subunit of the H1 and H2 subtypes could have arisen on the assumption that H2 evolved from the H1 subtype in a 42-yr period. But surveillance of human influenza strains since their isolation in 1933 (ref. 25) suggests that the H1 subtype did not undergo such an extensive 'drift', at least in man. No intermediate linking strains spanning the H1 and H2 subtypes were isolated; the H2 subtype appeared suddenly in 1957 (along with a new neuraminidase subtype) presumably from a mammal or bird reservoir in which influenza is common²⁶. There remains the possibility that drift of the H1 subtype giving rise to the H2 subtype occurred in a mammalian or avian reservoir. Indeed, H1 strains do infect pigs²⁵ but again no evidence of intermediate strains between the H1 and H2 subtype have been observed in the last 48 yr.

Thus, the H1 and H2 subtypes are evolutionary more closely related to one another than either is to the H3 and H7 subtypes but the evolutionary time scale cannot yet be estimated. It will be interesting to sequence completely some of the eight other known mammalian or avian haemagglutinin molecules because distant relationships between subtypes could have remained undetected in the traditional immunological screening methods used to classify haemagglutinin subtypes². An independent

Table 1 Conservation of amino acid and nucleotide sequences in strains of different H subtypes

	% Amino acid conservation			
	H1	H2	H3	H7
H1		58(79)	35(53)	33(51)
H2	61(72)		36(50)	35(53)
H3	45(58)	45(57)		36(65)
H7	44(58)	46(59)	45(66)	
% Nucleotide conservation				

The conservation in the HA1 subunit is given first, with the conservation in the HA2 subunit shown in parentheses. Amino acid conservation is listed above the diagonal and nucleotide conservation below. See Fig. 1 legend for the specific strains used in this comparison.

sequence analysis of the haemagglutinin of another H1 subtype, A/WSN/33, has recently been completed²⁷. This strain also shows homology to the H2 subtype.

We thank Mrs M. A. Robertson for supplying virus and RNA and Dr A. Caton for critical reading of the manuscript. G.G.B. is supported by a MRC project grant S.F. by a NSF predoctoral fellowship and G.W. by a research fellowship from Trinity College, Cambridge.

Note added in proof: We recently learnt of a Chinese report³³ providing serological evidence of shared antigenic determinants between late variants of the H1 subtype and early variants of the H2 subtype; this correlates well with their molecular homology.

Received 28 January; accepted 1 April 1981.

1. Tyrrell, D. A. J. & Smith, J. W. G. *Br. med. Bull.* **35**, 77–85 (1979).
2. Schild, G. C. *et al.* in *Structure and Variation in Influenza Virus* (eds Laver, W. G. & Air, G. M.) 373–383 (Elsevier, New York, 1980).
3. Porter, A. G. *et al.* *Nature* **282**, 471–477 (1979).
4. Gething, M. J. *et al.* *Nature* **287**, 301–306 (1980).
5. Both, G. W. & Sleight, M. J. *Nucleic Acids Res.* **8**, 2561–2575 (1980).
6. Min Jou, M. *et al.* *Cell* **19**, 683–696 (1980).
7. Sleight, M. J. *et al.* in *Structure and Variation in Influenza Virus* (eds Laver, W. G. & Air, G. M.) 69–78 (Elsevier, New York, 1980).
8. Verhoeven, M. *et al.* *Nature* **286**, 771–776 (1980).
9. Ward, C. W. & Doppeide, T. *Virology* **103**, 37–53 (1980).
10. Winter, G. & Fields, S. *Nucleic Acids Res.* **8**, 1965–1974 (1980).
11. Fields, S., Winter, G. & Brownlee, G. G. *Nature* **290**, 213–217 (1981).
12. Winter, G. *et al.* *Nucleic Acids Res.* **9**, 237–245 (1981).
13. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
14. Sanger, F. *et al.* *J. molec. Biol.* **143**, 161–178 (1980).
15. Hamlyn, P. H. *et al.* *Cell* **15**, 1067–1075 (1978).
16. Simoncsitz, A. *et al.* *Nature* **269**, 833–836 (1977).
17. Brownlee, G. G. in *Structure and Variation in Influenza Virus* (eds Laver, W. G. & Air, G. M.) 385–390 (Elsevier, New York, 1980).
18. Waterfield, M. D. *et al.* *Br. med. Bull.* **35**, 57–63 (1979).
19. Waterfield, M., Scrase, G. & Skehel, J. *Nature* **289**, 422–424 (1981).
20. Ward, C. W. & Doppeide, T. A. *Br. med. Bull.* **35**, 51–56 (1979).
21. Nakamura, K., Bhowan, A. S. & Compans, R. W. *Virology* **107**, 208–221 (1980).
22. Laver, W. G., Gerhard, W., Webster, R. G., Frankel, M. E. & Air, G. M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1425–1429 (1979).
23. Wiley, D. C., Wilson, I. A. & Skehel, J. J. *Nature* **289**, 373–378 (1981).
24. Brownlee, G. G. in *Expression of Eucaryotic, Viral and Cellular Genes* (Academic, London, in the press).
25. Pereira, M. S. *Br. med. Bull.* **35**, 9–14 (1979).
26. Laver, W. G. & Webster, R. G. *Br. med. Bull.* **35**, 29–33 (1979).
27. Hiti, A. L., Davis, A. R. & Nayak, D. P. *Virology* (in the press).
28. Gronenborn, B. & Messing, J. *Nature* **272**, 375–377 (1978).
29. Rothstein, R. J. *et al.* *Meth. Enzym.* **68**, 101–110 (1979).
30. Messing, J., Crea, R. & Seeburg, P. H. *Nucleic Acids Res.* **9**, 309–321 (1981).
31. Staden, R. *Nucleic Acids Res.* **8**, 3673–3694 (1980).
32. Duckworth, M. L. *et al.* *Nucleic Acids Res.* **9**, 1691–1706 (1981).
33. Gengqi, L. *et al.* *Scientia Sinica* **23**, 1063–1069 (1979).

The origins of replication of the yeast mitochondrial genome and the phenomenon of suppressivity

Miklos de Zamaroczy, Renzo Marotta, Godeleine Faugeron-Fonty, Regina Goursot, Marguerite Mangin, Giuseppe Baldacci & Giorgio Bernardi

Laboratoire de Génétique de Moléculaire, Institut de Recherche en Biologie Moléculaire, 2, Place Jussieu, 75005 Paris, France

The 'petite colonie' mutation of *Saccharomyces cerevisiae*^{1,2} is characterized by an irreversible loss of respiration and by an extraordinarily high spontaneous mutation rate^{3,4}. Crosses of wild-type cells with petite mutants exhibit a non-mendelian segregation of the mutation, yielding either wild-type progeny only^{1,2}, or both wild-type and petite mutants in proportions essentially dependent on the particular petite used³. In the first case, the petites entering the cross are called neutral, in the second one suppressive³. While the molecular basis of the spontaneous petite mutation is now understood^{4–12}, suppressivity has remained an elusive phenomenon for the past 25 yr. We report here that the mitochondrial genome of most spontaneous petites (which is exclusively made up by the tandem repetition of a DNA segment excised from the genome of parental wild-type cells^{5–8}) carries at least one of the *ori*

sequences of the parental wild-type genome. These are long homologous DNA stretches showing striking similarities with the origins of replication of mitochondrial DNAs from mammalian cells. The properties (intact or altered primary structure, high or low number) of the *ori* sequences of petite genomes seem to determine suppressivity—the level of transmission of petite genomes to the progeny of crosses with wild-type cells. These results indicate that *ori* sequences are indeed origins of DNA replication and that suppressivity depends on the relative replication efficiencies of petite and wild-type genomes.

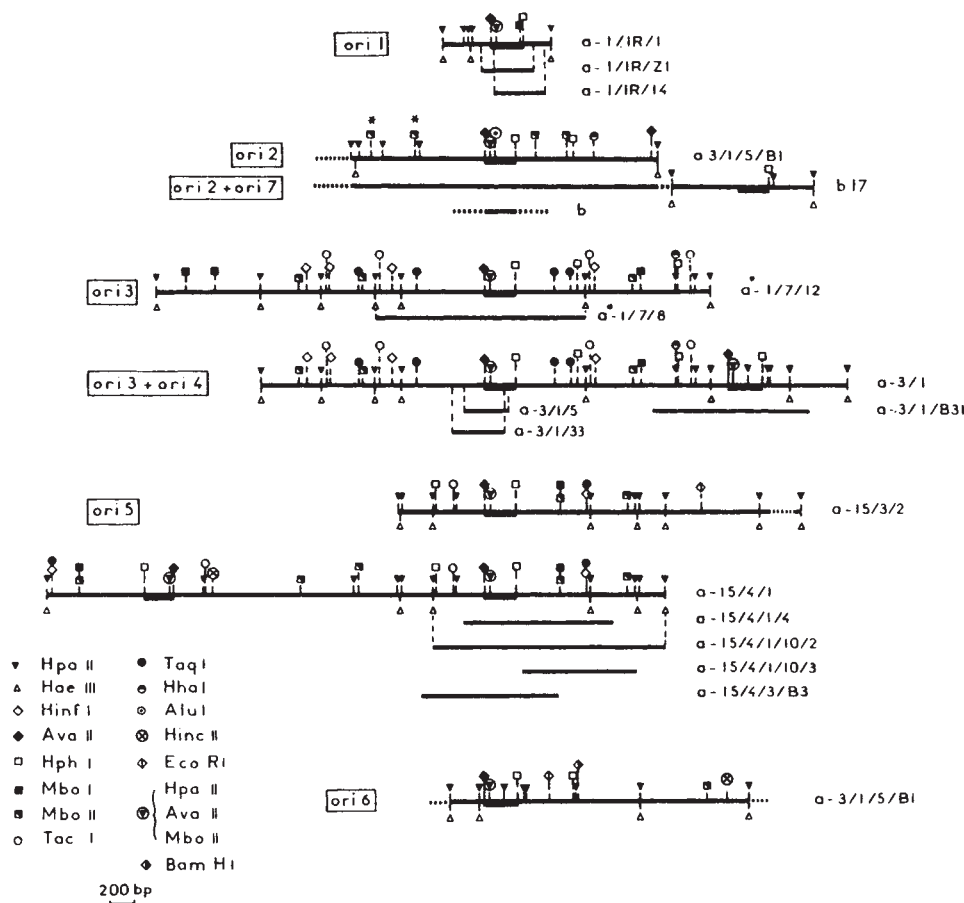
We previously proposed that the mitochondrial genome of wild-type yeast cells contains several origins of replication and that at least one of them is carried by the excised segment which will become the repeat unit of a spontaneous petite¹³. We tentatively localized such an *ori* sequence within the common central stretch of the genome of two spontaneous petites excised from the same region of the wild-type genome^{6,7}. This stretch is characterized (refs 7, 12 and Figs 1, 2, 4) by two short GC clusters, A and B, flanking a 23-nucleotide AT palindrome, *p*, and a short sequence, *s*; and by one long GC cluster, C, separated from B by long AT sequence, *l*. We then found a common 80-base pair sequence centred on cluster B, and more recently, the whole *ori* sequence in petite genomes derived from five regions of the wild-type genome^{10–12,14,15}.

Screening of the mitochondrial genomes of ~400 spontaneous petites derived from wild-type strain A⁵ revealed that all except two contained an *ori* sequence, as judged from the restriction sites located on clusters A, B and C (Fig. 1; Table 1). Restriction mapping showed that the *ori* sequences of different petite genomes were surrounded by different flanking regions and indicated the existence of at least seven *ori* sequences, *ori* 1–7 (Fig. 1), on the wild-type genome. This conclusion was confirmed by hybridization experiments in which labelled mitochondrial DNAs from petite genomes carrying different *ori* sequences showed seven common hybridization bands on *Hae*III fragments from the parental wild-type genome; these fragments had the same sizes as *Hae*III fragments carrying the different *ori* sequences on the petite genomes of Fig. 1. An eighth hybridization band might indicate the existence of yet another origin.

Figure 1 also shows that (1) three petite genomes, a-3/1/5/B1, b17 and a-3/1, contained two *ori* sequences each; (2) two petite genomes, a-3/1/5 and a-3/1/33, had an *ori* sequence lacking cluster C, and two petite genomes, a-1/1R/14 and a-1/1R/1/26, had an *ori* sequence lacking cluster A (*ori*⁻ petites, having a partially deleted *ori* sequence); (3) petite a-15/4/1 contained two *ori* sequences in opposite orientations—the only rearranged repeat unit found so far among spontaneous petites (this sort of petite will be indicated as *ori*⁺); (4) petite a-15/4/1/10/3, derived by subcloning from the previous one, lacked an *ori* sequence altogether; another *ori*⁰ petite, a-3/1/B4, was a diploid petite issued from a cross of a-3/1 with wild-type strain B and derived from the 15S-*oxi* 3 region of the B genome; these *ori*⁰ petite genomes will be discussed in detail elsewhere.

Figure 2 presents *ori* sequences 1–5 as determined on the repeat units of 11 different spontaneous petites (see Table 1). The *ori* 6 sequence and the right end of *ori* 7 were found in the published sequences of petites DS-400/A12 and DS-14 (refs 16, 17). Homology in the primary structure of different petite genomes carrying *ori* 1 or 3 was nearly perfect, the very rare differences being indicated by asterisks in Fig. 2. Comparison of different *ori* sequences indicated perfect homology in clusters A, B and C (some differences in clusters A and B of *ori* 6 being probably due to sequence uncertainties¹⁶ and several differences in region *l* between clusters B and C).

The stretch shared by all *ori* sequences is 265 base pairs long, its left border at nucleotide 69, and its right border at nucleotide 334 of Fig. 2. Note, however, that at the left of cluster A *ori* 6 presents a very large GC cluster, α , absent in at least *ori* 1–5; (2) *ori* 4 and 6 contain two additional GC clusters, β and γ , identical in both sequence and location; β is located between clusters B



and C, γ at the right of cluster C; at least cluster γ is also shared by *ori* 7; (3) *ori* 4–7 are homologous for 30–40 nucleotides at the right of the position of cluster γ ; (4) some sequence homology is present on the left of the *ori* sequences, particularly in *ori* 1 and 2; (5) the overall homology between *ori* 4, 6 and possibly 7 may comprise 370 nucleotides; (6) *ori* 1 and 2 show homologies for at least 30 base pairs on the right of the *ori* sequence; and (7) the full sequence of the repeat units of *ori* petites (ref. 12 and paper in preparation) indicated that these are the result of excisions involving two direct repeats, one of which is within the *ori* sequence.

Hybridization of labelled petite mitochondrial DNAs containing different *ori* sequences on restriction fragments from wild-type DNAs has been performed and restriction maps of both sets of DNAs have been compared with maps from other laboratories^{16–26}. Using these results, it was possible to localize and orient (Fig. 3) *ori* 1–7 on the mitochondrial genome of wild-type cells and show that the inverted repetition of *ori* 5 on a-15/4/1 was not present on the wild-type genome. The distribution of the *ori* sequences is very uneven with five sequences on one-third of the map, the region most often represented among the genomes of spontaneous petites²⁷. Note that *ori*

Fig. 1 Restriction enzyme maps of the repeat units of mitochondrial genomes of spontaneous petite mutants. These arose from wild-type strain A, except for a-3/1/5/B1, b17 and b, which derived from a region (*ori* 1–2) of wild-type strain B in which this genome has the same map as A. Some maps present additions and corrections compared with ref. 5. The map of b17 is a preliminary one; that of b contains an internal deletion in its repeat unit (in preparation). Restriction sites are indicated on some repeat units only and not all *Ava*II sites are shown. Asterisks indicate restriction sites whose locations need to be confirmed. *Ori* sequences are underlined: cluster A corresponds to an *Ava*II site, cluster B to *Hpa*II, *Ava*II, *Mbo*II site clusters, cluster C to a *Hph*I and a *Mn*I site. All maps are centred on cluster B and oriented with clusters A, B, C, from left to right. Vertical broken lines indicate excision sites where precisely known. bp, Base pairs.

Fig. 2 Primary structure of the *ori* sequences and their flanking regions, as determined by the method of Maxam and Gilbert⁴³ on the repeat units of the mitochondrial genomes from petites a-1/1R/1 (*ori* 1; position 1 corresponds to position 301 on the repeat unit of the reference sequence of a-1/1R/1), b (*ori* 2), a-3/1 (*ori* 3), a-3/1/B31 (*ori* 4), a-15/4/1/3/B3 (*ori* 5), DS-400/A12 (*ori* 6; from ref. 16) and DS-14 (*ori* 7; from ref. 17). The sequence on the left of *ori* 5 is from HS 1948 (ref. 31). *Ori* sequences were also determined on the repeat units of petites a-1/1R/Z1, a-1/1R/14 (*ori* 1) a-1/1R/26, a*-1/7/12, a*-1/7/8, a-3/1/5, a-3/1/33 (*ori* 3). Asterisks indicate the positions of modified nucleotides (indicated as N) or of differences in the same *ori* sequence as determined on different petites. Regions of *ori* sequences are indicated by thick lines for GC clusters A, B and C and thin lines for AT regions *p*, *s* and *l*. The positions of clusters β and γ in *ori* 4, 6 and 7 are given as well as the sequences of these clusters (bottom lines). Restriction sites are indicated by the symbols shown. The sequence of *ori* 3 corrects some mistakes¹⁴ in the 10 nucleotides at the left of cluster A, and in one nucleotide at the right of cluster B.

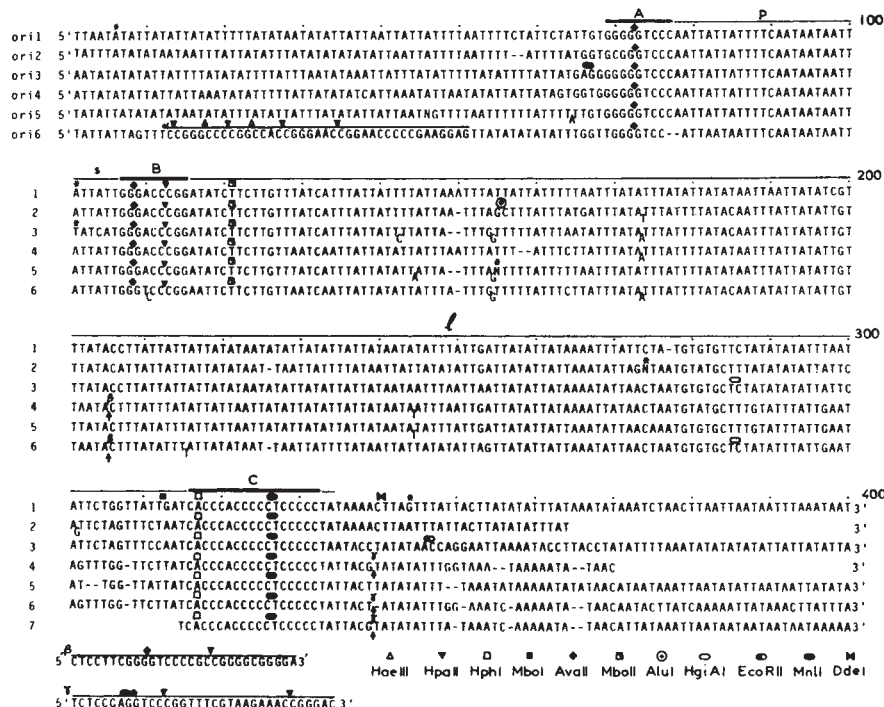


Table 1 The mitochondrial genomes of spontaneous petites: properties of *ori* sequences and suppressivity

Petite strain	Repeat unit length (base pairs)	Primary structure	<i>Ori</i> sequence	%Suppressivity	Transmission of petite genomes
a-1/1R/1	884	w	<i>ori</i> 1	>95	8:4
a-1/1R/Z1	416	w	<i>ori</i> 1	>95	
a-1/1R/40	606	m	<i>ori</i> 1	>95	
a-1/1R/14	380	w	<i>ori</i> 1 ⁻ (A ⁻)	80	11:0
a-1/1R/1/26	392	w	<i>ori</i> 1 ⁻ (A ⁻)	80	5:0
b	875	o	<i>ori</i> 2	>95	10:0
a*-1/7/12	4,500	o	<i>ori</i> 3	60-80	36:0
a*-1/7/8	1,760	o	<i>ori</i> 3	85	14:0
a-3/1	4,700	o	<i>ori</i> 3+4	60-80	28:4
a-3/1/5	345	w	<i>ori</i> 3 ⁻ (C ⁻)	<5	5:1
a-3/1/33	400	w	<i>ori</i> 3 ⁻ (C ⁻)	<5	
a-3/1/B31*	1,360	o	<i>ori</i> 4	—	—
a-15/3/2	4,300	m	<i>ori</i> 5	50-60	12:0
a-15/4/1	4,800	m	<i>ori</i> 5+5	<5	3:12
a-15/4/1/1	1,560	m	<i>ori</i> 5	85	12:0
a-15/4/1/4	1,220	m	<i>ori</i> 5	90	12:0
a-15/4/1/10/1	1,780	m	<i>ori</i> 5	90	12:0
a-15/4/1/10/2	1,830	m	<i>ori</i> 5	85	12:0
a-15/4/1/10/3	970	m	<i>ori</i> 5 ⁰	~1	5:13
a-15/4/1/B3*	1,170	o	<i>ori</i> 5	—	—
a-3/1/B/B1*	16,500	m	<i>ori</i> 2+6	—	—
b17	~7,800	—	<i>ori</i> 2+7	80	—

w and o indicate that the primary structure is known for the whole repeat unit or for the *ori* sequence and the flanking sequences, respectively. All other repeat units were physically mapped (m). The transmission of petite genomes concerns ~200 diploid petites from crosses of spontaneous petites with wild-type strain B (A in the case of petite b). Mitochondrial DNAs were mapped with restriction enzymes. The ratio presented is that of diploids having the repeat units of the parental petite to diploids having modified repeat units (see text).

* Diploid petites from crosses of spontaneous petites with wild-type cells. The repeat units of a-3/1/B31, a-15/4/1/B3 and a-3/1/5/B1 originated from the genomes of a-3/1, a-15/4/1 and B, respectively. They were used to sequence *ori* 4, *ori* 5 and to map *ori* 6, respectively.

sequences do not have the same orientation (Fig. 3) and that *ori* 3 and 4, and *ori* 2 and 7, share a very similar tandem arrangement.

Three independent lines of evidence indicate that the *ori* sequences are indeed origins of replication. (1) The region between clusters A and B inclusive can be folded in a way (Fig. 4) very similar to that reported for a region of the replication origin of mammalian mitochondrial DNA²⁸⁻³⁰; all base pair changes found in this region in different genomes are localized in the looped-out sequence *s*. Cluster C is almost identical (Fig. 4) in sequence to a GC cluster, similarly located relative to the A-B region, in mammalian mitochondrial DNAs²⁸⁻³⁰. (2) *Ori* sequences are present in the repeat units of most spontaneous petites. (3) A clear correlation exists between the properties of the *ori* sequences and suppressivity, as indicated by the following.

We reported previously that crosses of two spontaneous petites (a-1/1R/1 and b) with wild-type cells produced diploids only harbouring the unaltered mitochondrial genome of the parental petite used in the cross^{10-12,14,15}. Both petites were very highly suppressive (that is, the percentage of petites in the progeny was >95%) and had mitochondrial genomes characterized by very short repeat units (~1% of the wild-type genome). We called these petites, which have also been reported from other laboratories^{31,32}, supersuppressive, and suggested that their mitochondrial genomes were only ones found in the progeny because they contained multiple copies of the origin of replication due to tandem amplification of their repeat units and they replicated more efficiently than the wild-type genome, which they could compete out^{10-12,14,15}. We have now investigated the general case of petites having any degree of suppressivity. The results are presented in Table 1 and lead to the following conclusions.

(1) Most of the 200 petite diploids had restriction maps identical to those of the parental petites, regardless of the suppressivity of the latter. The few cases where a different situation was found (Table 1) corresponded either to a genome resulting from a new excision process which had occurred in the parental petite genome or, more rarely, in the parental wild-type genome.

(2) Partial or total deletions or rearrangements of *ori* sequences were always accompanied by drastic changes in suppressivity: the total deletion of *ori* 5 in a-15/4/1/10/3 resulted in a suppressivity of ~1% as opposed to 90% for

a-15/4/1/4; the deletion of cluster C plus about 100 base pairs on its left in *ori* 3 led to a suppressivity <5% in a-3/1/5 and a-3/1/33, whereas a*-1/7/8 (although having a much larger repeat unit) had a suppressivity of 85%; the deletion of cluster A in *ori* 1 caused a decrease in suppressivity, from >95% in a-1/1R/Z1 to 80% in a-1/1R/14 and a-1/1R/1/26; and the presence of a duplicated inverted *ori* 5 sequence in a-15/4/1 was associated with a suppressivity of ~5%, whereas a-15/3/2, having a comparable repeat length, but only one *ori* 5 sequence, had a suppressivity of 50-60%.

(3) The repeat unit length affects replication efficiency as seen by comparing the suppressivities of a*-1/7/8 and a*-1/7/12, two petites carrying the same *ori* 3 sequence, or of a-15/3/2 and the series a-15/4/1, all containing *ori* 5. In all cases a shorter repeat length, and thus a higher density of *ori* sequences, is accompanied by a higher suppressivity. Similar observations have been reported previously³.

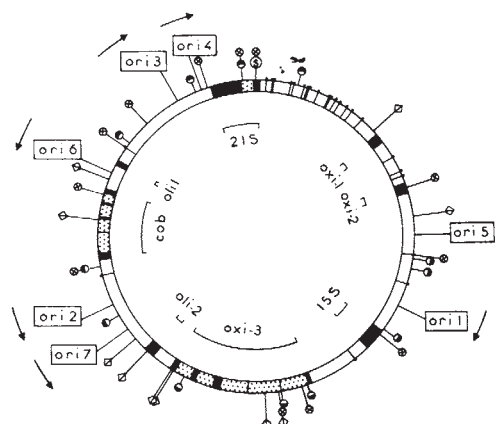


Fig. 3 Localization of *ori* sequences on the physical map of the mitochondrial genome of wild-type strain A. (This map is almost identical to that of strain KL-14-4A¹⁸.) Positions indicated correspond to cluster B. Restriction sites are indicated by the symbols used in Fig. 1; Ⓢ corresponds to the *Sal*I site, used as the map origin. The orientation of *ori* sequences is given by arrows pointing in the direction of cluster C. Black and hatched areas correspond to exons and introns, respectively, of mitochondrial genes. Their localization is based on refs 16-26. The localization of tRNA genes is shown by thin radial lines.

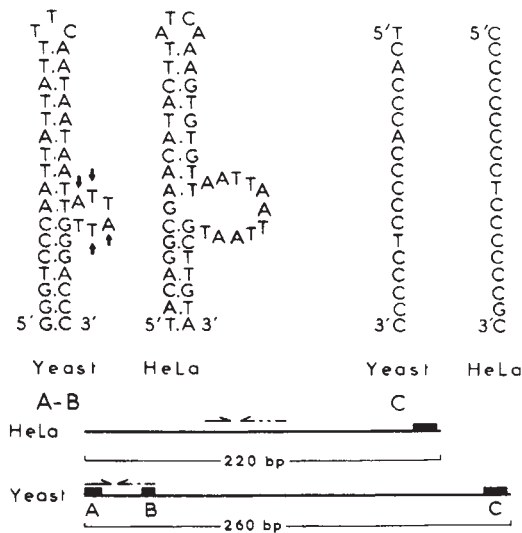


Fig. 4 Comparison of *ori* sequences of mitochondrial genomes from yeast (present work) and HeLa cells²⁸. Homology of potential secondary structure is found for the inverted repeats in the A-B region (arrows indicate the base changes found in this region in different petite genomes). Homology of primary structure is found for cluster C. The bottom compares the two *ori* sequences; the arrows indicate the inverted repeats of the A-B region, the broken line corresponding to the looped-out sequence. bp, Base pairs.

Two explanations have been put forward to account for suppressivity. The first one proposes a replicative advantage of the mitochondrial genome of suppressive petites over that of wild-type cells³³⁻³⁶. It was directly inspired by the work of Mills *et al.*³⁷ on the replication of Q β DNA but was not accompanied by any molecular model. The second one proposes a destructive recombination of the petite genome with the wild-type genome³⁸⁻⁴², and predicts that a number of different petite genomes are formed as the consequence of the increased parental genome instability due to the insertion of the petite genome. The present results contradict this latter explanation because most of the diploid petites studied here had genomes identical to those of the parental petites. Indeed, they provide for the first time a precise molecular basis for the former explanation of the replicative competition.

We thank Miss Claire Gaillard, Dr René Goursot and Mr Alain Huyard for help in mapping and sequencing studies, Miss Martine Brient for typing this manuscript and Mr Philippe Breton for the artwork.

Received 12 January; accepted 20 March 1981.

1. Ephrussi, B. in *Unités Biologiques Douées de Continuité Génétique*, 165-180 (Editions du CNRS, Paris, 1949).
2. Ephrussi, B. in *Nucleocytoplasmic Relations in Microorganisms*, 13-47 (Clarendon, Oxford, 1952).
3. Ephrussi, B., de Margerie-Hottinguer, H. & Roman, H. *Proc. natn. Acad. Sci. U.S.A.* **41**, 1065-1071 (1955).
4. Bernardi, G. *Trends biochem. Sci.* **4**, 197-201 (1979).
5. Faugeron-Fonty, G. *et al. J. molec. Biol.* **134**, 493-537 (1979).
6. Gaillard, C. & Bernardi, G. *Molec. gen. Genet.* **174**, 335-337 (1979).
7. Gaillard, C., Strauss, F. & Bernardi, G. *Nature* **283**, 218-220 (1980).
8. Baldacci, G., de Zamaroczy, M. & Bernardi, G. *FEBS Lett.* **114**, 234-236 (1980).
9. Bernardi G. & Bernardi, G. *FEBS Lett.* **115**, 159-162 (1980).
10. Bernardi, G. *et al. in DNA Recombination, Interactions and Repair* (eds Zadrzilk, S. & Sponar, J.) 77-84 (Pergamon, Oxford, 1980).
11. Bernardi, G. *et al. in Mobilization and Reassembly of Genetic Information* (eds Scott, W. A., Werner, K., Joseph, D. R. & Schultz, J.) 119-132 (Academic, New York, 1980).
12. Bernardi, G. *et al. in The Organization and Expression of the Mitochondrial Genome* (eds Kroon, A. M. & Saccone, C.) 21-31 (Elsevier, Amsterdam, 1980).
13. Prunelli, A. & Bernardi, G. *J. molec. Biol.* **110**, 53-74 (1977).
14. de Zamaroczy, M., Baldacci, G. & Bernardi, G. *FEBS Lett.* **108**, 429-432 (1979).
15. Goursot, R., de Zamaroczy, M., Baldacci, G. & Bernvardi, G. *Curr. Genet.* **1**, 173-176 (1980).
16. Nobrega, F. G. & Tzagoloff, A. *J. biol. Chem.* **255**, 9828-9837 (1980).
17. Macino, G. & Tzagoloff, A. *Cell* **20**, 507-517 (1980).
18. Sanders, J. P. M., Heyting, C., Verbeet, M. Ph., Meijlink, F. C. P. W. & Borst, P. *Molec. gen. Genet.* **157**, 239-261 (1977).
19. Wesolowski, M., Monnerot, M. & Fukuhara, H. *Curr. Genet.* **2**, 121-129 (1980).
20. Tabak, H. F., Hecht, N. B., Menke, M. M. & Hollenberg, C. P. *Curr. Genet.* **1**, 33-43 (1979).
21. Coruzzi, G. & Tzagoloff, A. *J. biol. Chem.* **254**, 9324-9330 (1979).
22. Heyting, C. *et al. Molec. gen. Genet.* **168**, 231-250 (1979).
23. Thalenfeld, B. E. & Tzagoloff, A. *J. biol. Chem.* **255**, 6173-6180 (1980).
24. Bonitz, S. G., Coruzzi, G., Thalenfeld, B. E. & Tzagoloff, A. *J. biol. Chem.* (in the press).
25. Tzagoloff, A., Nobrega, M., Akai, A. & Macino, G. *Curr. Genet.* **2**, 149-157 (1980).

26. Berlani, R. E., Bonitz, S. G., Coruzzi, G., Nobrega, M. & Tzagoloff, A. *Nucleic Acids Res.* **8**, 5017-5030 (1980).
27. Mathews, S., Schweyen, R. J. & Kaudewitz, F. in *Mitochondria 1977* (eds Bandlow, W. et al.) 133-138 (de Gruyter, Berlin, 1977).
28. Crews, S., Ojala, D., Possakony, J., Nishiguchi, J. & Attardi, G. *Nature* **277**, 192-198 (1979).
29. Gillum, A. M. & Clayton, D. A. *J. molec. Biol.* **135**, 353-368 (1979).
30. Kobayashi, M., Yagimura, K., Seki, T. & Koike, K. in *The Organization and Expression of the Mitochondrial Genome* (eds Kroon, A. M. & Saccone, C.) 221-229 (Elsevier, Amsterdam, 1980).
31. Blanc, H. & Dujon, B. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3942-3946 (1980).
32. Gingold, E. B. *10th Int. Conf. of Yeast Genetics and Molecular Biology*, Abstr. no 333, 143 (1980).
33. Carnevali, F., Morpurgo, G. & Tecce, G. *Science* **163**, 1331-1333 (1969).
34. Rank, G. H. *Can. J. Genet. Cytol.* **12**, 129-136 (1970).
35. Rank, G. H. *Can. J. Genet. Cytol.* **12**, 340-346 (1970).
36. Rank, G. H. & Bech-Hansen, N. T. *Can. J. Microbiol.* **18**, 1-7 (1972).
37. Mills, D. R., Peterson, R. L. & Spiegelman, S. *Proc. natn. Acad. Sci. U.S.A.* **58**, 217-224 (1967).
38. Coen, D., Deutsch, J., Netter, P., Petrochilo, E. & Slonimski, P. P. in *Control of Organelle Development*, 24th Symp. Soc. exp. Biol., 449-496 (Cambridge University Press, London, 1970).
39. Deutsch, J. et al. *Genetics* **76**, 195-219 (1973).
40. Michaelis, G., Petrochilo, E. & Slonimski, P. P. *Molec. gen. Genet.* **123**, 51-65 (1973).
41. Perlman, P. S. & Birky, C. W. *Proc. natn. Acad. Sci. U.S.A.* **71**, 4612-4616 (1974).
42. Slonimski, P. P. & Lazowska, J. in *Mitochondria 1977* (eds Bandlow, W., Schweyen, R. J., Wolf, K. & Kaudewitz, F.) 39-52 (de Gruyter, Berlin, 1977).
43. Maxam, A. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560-564 (1977).

Structure of C-terminal half of two H-2 antigens from cloned mRNA

**F. Brégégère*, J. P. Abastado*, S. Kvist†, L. Rask*§,
J. L. Lalanne*, H. Garoff†, B. Cami*, K. Wiman‡,
D. Larhammar‡, P. A. Peterson‡, G. Gachelin*,
P. Kourilsky* & B. Dobberstein†**

*Unité de Biologie Moléculaire du Gène, ER CNRS no. 201 and
SCN INSERM no. 20, Institut Pasteur, 28 rue du Dr Roux,
75724 Paris Cédex 15, France

†European Molecular Biology Laboratory, Meyerhofstrasse 1,
Postfach 102 209, D-6900 Heidelberg, FRG

‡Department of Cell Research, The Wallenberg Laboratory,
University of Uppsala, Uppsala, Sweden

The classical cell-surface histocompatibility antigens (H-2 antigens in the mouse), known to have key roles in cell-to-cell recognition¹, are encoded by at least three highly polymorphic genes (*H-2D*, *K* and *L*)². Like their human (HLA) counterparts³, H-2 heavy chains span the cell membrane with a short C-terminal cytoplasmic region and an N-terminal extracellular stretch of about 280 amino acids. HLA antigens seem to be organized in three domains containing β -pleated sheets, with disulphide loops within the second and third domains, but the relative scarcity of material has hampered biochemical studies of the H-2 antigens⁴⁻⁶. We now report the sequencing of plasmids carrying H-2 cDNA as a means of inferring the amino acid sequence of the antigens, and especially of their previously poorly described C-terminal half.

The isolation of recombinant plasmids pH-2^d-1 and pH-2^d-3 is described in Fig. 1 legend and elsewhere⁷. Restriction maps of the cDNA inserts, 1,150 and 980 base pairs (bp) long, respectively, are different, but can be tentatively aligned on *Pvu*II, *Sst*I and *Pst*I sites (Fig. 1). Both inserts contain a noncoding stretch of about 480 bp next to the poly(A) sequence. The 627- and 479-bp long coding sequences and their corresponding amino acid sequences are given in Fig. 2. They show extensive homologies with available sequences of H-2 and HLA molecules (82% with H-2K^b, 73% with HLA B7) (Fig. 3)^{3-6,8-11}, allowing unequivocal alignment in the third domain. With reference to HLA³, we assigned nucleotide 133 to the first tryptophan residue in pH-2^d-1 and nucleotide 181 to the first arginine in pH-2^d-3. Both clones should, accordingly, code for the entire third domain, the membrane spanning region and the cytoplasmic segment.

§Present address: Department of Cell Research, The Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden.

Fig. 1 Restriction maps of pH-2^d-1 and pH-2^d-3 inserts and strategies to sequence them. The cDNA library from which pH-2^d-1 (ref. 7) was selected had been constructed using mRNA from SL2 lymphoma cells grown as ascites in DBA/2 mice (H-2^d haplotype). The 400 independent bacterial clones of this library were further screened by *in situ* hybridization²⁹, using a fragment of the insert of the first H-2 clone as a probe. The DNAs of the positive responders were then tested for the specific binding of H-2 mRNA as already described⁷. At least two of them, pH-2^d-2 and pH-2^d-3, were found positive in this test. The cDNA insert of pH-2^d-2 was found identical to part of that of pH-2^d-1, and was not analysed further.

Plasmid DNA was prepared from cleared lysates³⁰, partially purified by centrifugation in a CsCl/ethidium bromide gradient, and further purified by fractionation through a 5–40% sucrose gradient³¹. Digestion with restriction endonucleases (Biolabs, Boehringer or BRL) were carried out in standard conditions. Restriction maps were constructed from the size of the DNA fragments, estimated from electrophoretic patterns on agarose or acrylamide gels^{32,33}. As indicated, each cDNA insert is bordered by two reconstituted *Pst*I sites³⁴. Both inserts have the same orientation with respect to pBR322 map. pBR322 sequences are presented here in their usual orientation, so that the parts of the cDNA sequences corresponding to the 3' ends of the messengers are on the left-hand side of the inserts. The coding regions are represented by thick lines. The restriction fragments sequenced are represented by arrows. They were labelled at their 5' (●) or 3' (◆) ends, and cleaved secondarily to generate subfragments with only one labelled end³⁵. Sequencing techniques used were those of Maxam and Gilbert³⁵ (—) or Maat and Smith³⁶ (---).

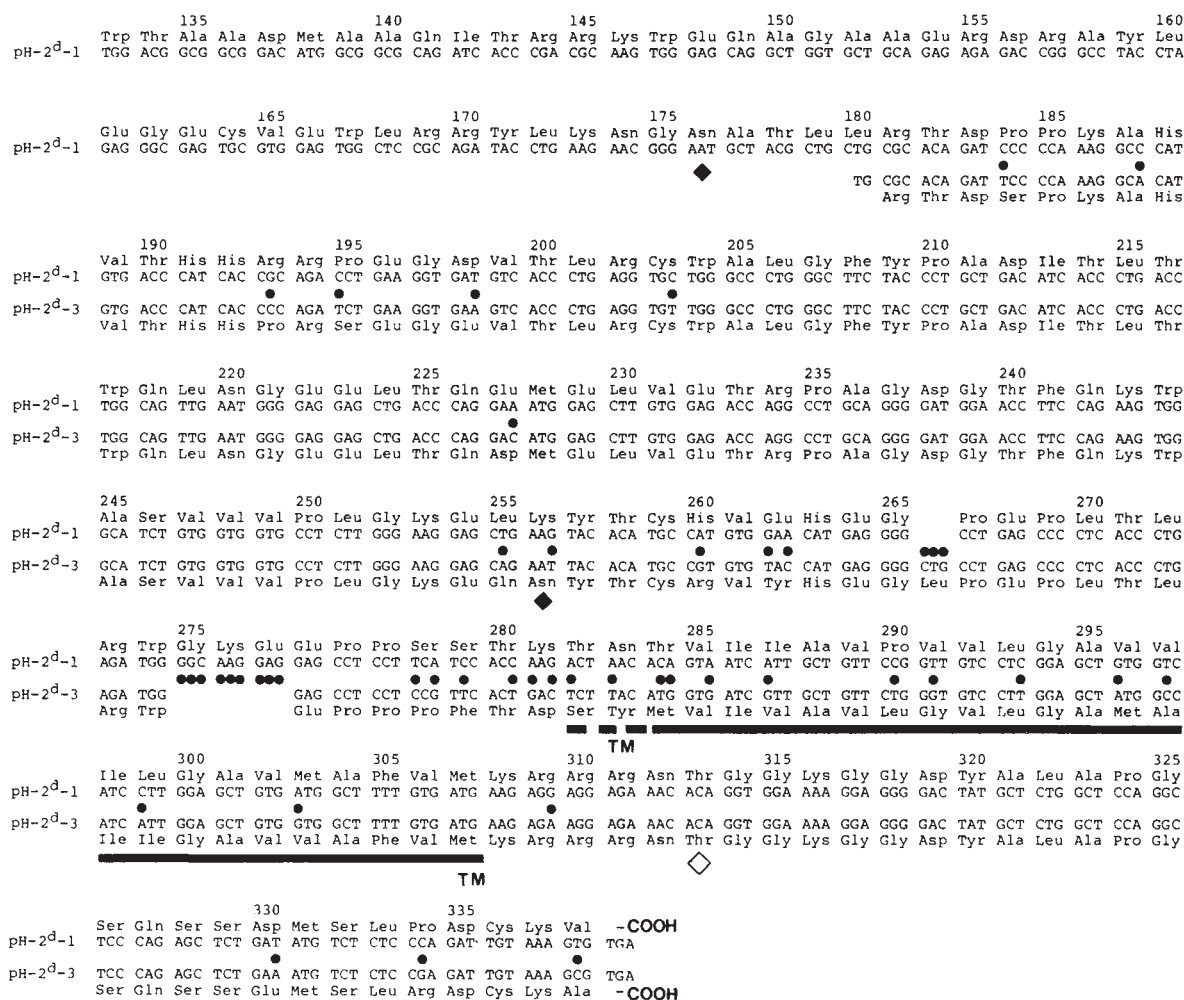


Fig. 2 Coding sequences of pH-2^d-1 and pH-2^d-3. Both sequences have been aligned as described in the text, the 5' terminus is to the left, the 3' terminus to the right. The putative glycosylation (◆) and phosphorylation (◐) sites are labelled. The positions at which a difference is found between the two nucleotide sequences are labelled (●).

A continuous stretch of 26 uncharged amino acids, mostly hydrophobic, extends from amino acids 282 to 307, displaying at the expected position the characteristic features of a membrane-spanning segment. Noticeably, it contains a repetition (Val-Val-Leu-Gly-Ala-Val, followed by Val-Ile-Leu-Gly-Ala-Val) in pH-2^d-1, also seen at the nucleotide level in pH-2^d-3. The amino acid residues differ in 10 out of the 26 positions, suggest-

ing that the major constraint is the sole maintenance of hydrophobicity. No homology with membrane-spanning segments of other membrane proteins was found.

Amino acids 308–338 correspond to intra-cytoplasmic sequences which have been reported to be phosphorylated¹² and associated with components of the cytoskeleton¹³. A possible phosphorylation site (Arg-Asn-Thr)¹⁴ is found at position 313 in

both H-2 clones. At the border with the membrane, a cluster of four basic residues (Lys-Arg-Arg-Arg) is found in both clones. As clusters of basic amino acids in similar positions have been found in HLA-A2 and HLA-B7 (ref. 15), membrane-bound IgM¹⁶, human glycoprotein¹⁷ and several viral glycoproteins¹⁸, we propose that they might be involved in the positioning of transmembrane proteins.

The amino acid sequence located at the external membrane border shows many variations. The conserved proline residues at positions 276–278 indicate breakage of the α -helical structure, suggesting that this segment can form a flexible link, in agreement with the accessibility of this region to papain⁶.

pH-2^d-1 codes for the third domain and half of the second, whereas pH-2^d-3 codes for the third domain only. Cysteine residues at positions 164, 203 and 269 are likely to be those involved in intrachain disulphide bridges, as they are in H-2K^b (refs 4, 5, 19). Possible glycosylation sites (Asn-Tyr-Thr)²⁰ are found at positions 176 and 256 in pH-2^d-1 and pH-2^d-3, respectively. The two amino acid sequences are extremely similar. Divergences are found mainly as clusters (positions 193–198, 225–227, 255–268, 275–303) also seen in comparisons with HLA with additional variations.

The third domain of HLA shares significant homologies with the constant domains of immunoglobulin heavy chains^{21,22}. Using the alignment frame designed for HLA²¹, we found that the third domains of the H-2 molecules encoded by the two plasmids display the same type of homology (in preparation). At 20 out of the 23 aligned positions corresponding to hydrophobic amino acids involved in the β -pleated sheet structure in immunoglobulins, hydrophobic residues are also found in H-2 sequences. These results suggest strongly that the third domain of H-2 antigens, like that of HLA, is folded in an immunoglobulin-like three-dimensional structure. When the aforementioned clusters of amino acid differences between pH-2^d-1 and pH-2^d-3 are placed in the three-dimensional immunoglobulin model, they fall in loop areas (in 8 differences out of 10), while β -pleated sheets correspond to highly conserved regions. This suggests that the three-dimensional structure of the third domain imposes constraints on divergences. This could be true for other parts of the molecule as well and be important in the understanding of the basis of alloantigenicity.

Comparisons with available data on H-2D^d, K^d and L^d (Fig. 3) show that pH-2^d-1 differs from H-2L^d at positions 155, 156, 169 and 262. It has a methionine at position 138, whereas the cyanogen bromide cleavage map of H-2K^d indicates that there is no such residue in the molecule²³. At 57 out of 58 assigned positions the pH-2^d-1 amino acid sequence is identical to that of H-2D^d, the only difference being at amino acid 255, denoted as 'tentatively assigned'¹⁰. Therefore, pH-2^d-1 cannot code for H-2L^d or H-2K^d, but could well code for H-2D^d. The pH-2^d-3

Table 1 Analysis of the nucleotide changes between pH-2^d-1 and pH-2^d-3 sequences

	Replacements	Silent substitutions	Total substitutions
Codons 183–284 (third domains)	17/704 = 0.024	5/214 = 0.023	22/918 = 0.024
Codons 285–308 (membrane spanning regions)	7/148.5 = 0.047	2/58.5 = 0.034	9/207 = 0.043
Codons 309–339 (cytoplasmic fragments)	3/221.5 = 0.014	1/66.5 = 0.015	4/288 = 0.014
Codons 183–339	27/1,074 = 0.025	8/339 = 0.024	35/1,413 = 0.025

pH-2^d-1 and pH-2^d-3 were compared over their aligned sequences (Fig. 2). The rate of 'silent substitutions' (see text) was determined by a computation similar to that described by Lomedico *et al.*²⁸: all possible single-step mutations (that is, three possible changes for each base) were totalled over the 157 aligned codons, and classified as replacements if they involved an amino acid change, and as silent substitutions if they did not. The numbers were then averaged for the two genes. The fractions displayed in the table indicate the number of replacements (or silent or total substitutions) actually recorded over the total number of possible replacements (or silent or total substitutions).

sequence differs from H-2D^d at position 262 (ref. 11) and is compatible with the 15 assigned positions reported for H-2L^d in the corresponding area¹¹. It has a possible glycosylation site²⁰ at positions 252–258 as would be expected for H-2K^d (P. Robinson, personal communication). Protein sequence data are thus too limited to allow conclusive assignments, especially as the cloned sequences could also specify Tla, Qa1, Qa2 or other H-2-like antigens²⁴, in line with the finding that the mouse genome contains multiple H-2-related sequences²⁵.

The nucleotide sequences of pH-2^d-1 and pH-2^d-3 diverge in only 47 (9.7%) of the 485 bases aligned for comparison (including 12 aligned with empty positions, Fig. 2). The third domain shows remarkable conservation with only one base change in a stretch of 156 nucleotides (positions 204–255). Surprisingly, silent changes (with no corresponding amino acid change) are unusually rare, compared with replacement changes (Table 1), whereas in other genes the former arise more often than the latter^{26,27}. This raises several hypotheses: a special conservative constraint might exist on the nucleotide sequences themselves, making the silent changes not neutral. Alternatively, the two proteins may have diverged too rapidly to allow the accumulation of neutral mutations in their genes²⁶. Whether this feature is related to a mechanism involved in the generation of the natural polymorphism of these genes or not remains to be investigated.

We thank Miss O. Lebal for technical assistance, Mr B. Caudron for assistance in computer usage and Dr D. Meyer for a critical reading of the manuscript. The work described here was done in accordance with the French and German guidelines for

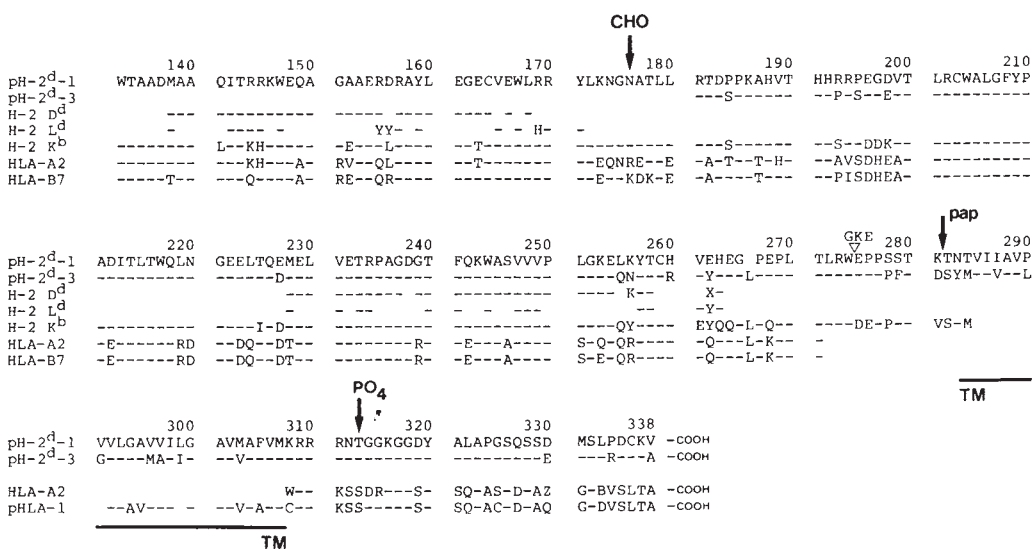


Fig. 3 Comparison of the amino acid sequences encoded by pH-2^d-1 and pH-2^d-3 with sequences of murine and human histocompatibility antigens^{3,6,8-11,15,23}. pHLA-1 is a recombinant plasmid carrying an HLA cDNA sequence³⁷. Its deduced amino acid sequence shows no difference from the published data on HLA-B7 COOH-terminal fragment¹⁵. The amino acids are indicated according to the one-letter code³⁸. pH-2^d-1 insert has been taken as a reference to align the other sequences. Three residues have been taken out of the alignment between positions 274 and 275, as indicated, to keep to numbering conventions already used for H-2 and HLA sequences⁵. The dashes indicate identity with pH-2^d-1 sequence. X indicates an undetermined amino acid, different from tyrosine.

recombinant research. This work was supported by grants to P.K. from CNRS (ER 201 and ATP 4246), INSERM (SCN 20 and CRAT 72-79-104) and DGRST (79-7-0500 and 78-7-2931), to G.G. from DGRST (79-7-0790) and to P.P. from the Swedish Cancer Society.

Received 12 March; accepted 8 May 1981.

- Zinkernagel, R. M. & Doherty, P. C. *Adv. Immun.* **27**, 51–177 (1979).
- Klein, J. *Science* **203**, 516–521 (1979).
- Strominger, J. L. *Prog. Immun.* **4**, 541–554 (1980).
- Uehara, H. *et al. Biochemistry* **19**, 306–315 (1980).
- Uehara, H. *et al. Biochemistry* **19**, 6182–6188 (1980).
- Martinko, J. A. *et al. Biochemistry* **19**, 6188–6193 (1980).
- Kvist, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 2272–2276 (1981).
- Orr, H. T., Lopez De Castro, J. A., Lancet, D. & Strominger, J. L. *Biochemistry* **18**, 5711–5720 (1979).
- Orr, H. T., Lopez De Castro, J. A., Parham, P., Ploegh, H. L. & Strominger, J. L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4395–4399 (1979).
- Nairn, R., Nathenson, S. G. & Coligan, J. E. *Eur. J. Immun.* **10**, 495–503 (1980).
- Coligan, J. E. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 1134–1138 (1980).
- Pober, J. S., Guild, B. C. & Strominger, J. L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 6002–6006 (1978).
- Geiger, B. & Singer, S. J. *Cell* **16**, 213–222 (1979).
- Kemp, D. E., Graves, D. J., Benjamini, E. & Krebs, E. G. *J. biol. Chem.* **252**, 4888–4894 (1977).
- Robb, R. J., Terhorst, C. & Strominger, J. L. *J. biol. Chem.* **253**, 5319–5324 (1978).
- Rogers, J. *et al. Cell* **20**, 303–312 (1980).
- Tomita, M. & Marchesi, V. T. *Proc. natn. Acad. Sci. U.S.A.* **72**, 2964–2968 (1975).
- Wickner, W. *Science* **210**, 861–868 (1980).
- Ewenstein, B. M. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 2909–2913 (1978).
- Marshall, R. D. *A. Rev. Biochem.* **41**, 673–709 (1972).
- Orr, H. T., Lancet, D., Robb, R. J., Lopez De Castro, J. A. & Strominger, J. L. *Nature* **282**, 266–270 (1979).
- Tragardh, L., Rask, L., Wiman, K., Fohlman, J. & Peterson, P. A. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1129–1133 (1980).
- Kimball, E. S., Maloy, E. S., Martinko, J. M., Nathenson, S. G. & Coligan, J. E. *Molec. Immun.* **17**, 1283–1291 (1980).
- Vitetta, E. S. & Capra, J. D. *Adv. Immun.* **26**, 147–193 (1978).
- Cami, B., Bregegere, F. & Kourilsky, P. *Nature* (in the press).
- Perler, F. *et al. Cell* **18**, 545–558 (1979).
- Miyata, T., Yasunaga, T. & Nishida, T. *Proc. natn. Acad. Sci. U.S.A.* **77**, 7328–7332 (1980).
- Lomedico, P. *et al. Cell* **18**, 545–558 (1979).
- Grunstein, M. & Hogness, D. S. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3961–3966 (1975).
- Bastia, D. J. *molec. Biol.* **124**, 601–639 (1978).
- Rougeon, F., Kourilsky, P. & Mach, B. *Nucleic Acids Res.* **2**, 2365–2378.
- Smith, H. O. & Birnstiel, M. L. *Nucleic Acids Res.* **3**, 2387–2398 (1975).
- Garoff, H., Frischauf, A. M., Simons, K., Lehrach, H. & Delius, H. *Nature* **288**, 236–241 (1980).
- Villa-Komaroff, L. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 3721–3727 (1978).
- Maxam, J. & Gilbert, M. *Meth. Enzym.* **65**, 499–560 (1980).
- Maat, J. & Smith, A. J. H. *Nucleic Acids Res.* **5**, 4537–4545 (1978).
- Ploegh, H. L., Orr, H. T. & Strominger, J. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6081–6085 (1980).
- IUPAC-IUB Commission on Biochemical Nomenclature *J. biol. Chem.* **243**, 3557–3559 (1968).

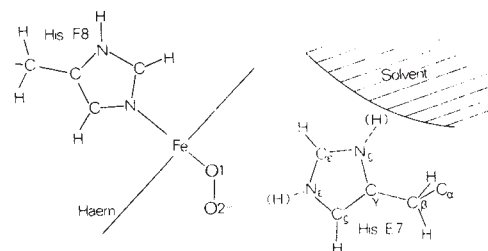


Fig. 1 Arrangement of proximal (F8) and distal (E7) histidines in oxyMb. At pH 8.4, nitrogen-bound hydrogen on E7 imidazole can be bonded to either N^ε (the naturally predominant form), with a hydrogen bond to O-2 (dotted line), or to N^δ, where it projects into the solvent surrounding the molecule.

Pauling² first proposed that the imidazole may form a hydrogen bond to the terminal oxygen atom (O-2 in Fig. 1), which carries a formal negative charge in his view of the electronic structure of the FeO₂ complex. Evidence suggesting such a bond comes from electron paramagnetic resonance and oxygen affinity data on cobalt-substituted Hb and Mb^{3–5}. The pK of the distal histidine is ~5.5 (ref. 4). At physiological pH (and the pD of the crystals used here) the histidine may have hydrogen-bonded to either N^ε or N^δ (see Fig. 1), and interaction with O-2 may therefore be by a hydrogen bond, or a simple van der Waals contact. X-ray crystallography of protein crystals cannot distinguish between these alternatives as hydrogen atoms scatter X rays only weakly, and are not normally visible in electron density maps. Neutrons, however, are scattered as strongly by hydrogen and deuterium as C, N, O, S and Fe atoms, and well-ordered H and D atoms may be observed in neutron density maps of proteins^{6,7}.

Crystals of oxyMb were prepared from frozen sperm-whale skeletal muscle¹. Large crystals (8 mm³) were transferred to deuterated mother liquor (pD 8.4) at 20 °C 3 months before data collection, because hydrogen gives strong incoherent scattering of neutrons which increases the background level in diffraction data collection. Replacement of H₂O solvent in crystals with D₂O, and subsequent replacement of exchangeable H atoms with D in the protein, alleviates this problem and improves the signal-to-noise ratio of the data. It also allows exchangeable H atoms to be identified in the density map, as H scatters out of phase with respect to D.

Neutron diffraction data were collected using the protein crystallography station of the High Flux Beam Reactor at Brookhaven National Laboratory. The diffractometer was equipped with a two-dimensional multiwire proportional counter⁸ and a cooling device to maintain the crystals at –5 °C and retard oxidation of the haem iron. Two crystals were used for data collection, each being exposed to the neutron beam for 21 days. No radiation damage or oxidation was observed; 88% of available data to 2 Å resolution was collected, together with further data between 2 and 1.5 Å, giving 14,411 independent reflections. The merging *R* factor between crystals was 14.1% on intensities.

Calculated phases and amplitudes for the neutron data were computed from the coordinates of all C, N, O, S and Fe atoms in the refined X-ray structure¹, including 60 ordered H₂O molecules. A difference density map (coefficients $|F_o| - |F_c|$: crystallographic *R* factor 35% for 10,152 reflections with $I > 1.5\sigma(I)$) showed clear peaks for 40% of the missing H and D atoms. Small peaks were visible at both N^ε and N^δ of His E7. H and D atoms observed in the map were added to the model, together with unobserved ones whose positions were known from stereochemistry (for example, most C–H groups), but ring nitrogen-bound H or D atoms for histidines were omitted—this reduced *R* to 33%. A second difference map failed to resolve the ambiguity at His E7, and combined crystallographic and conformational energy refinement was initiated, using methods described for X-ray refinement of oxyMb, but modified for use with neutron data. Seven cycles of coordinate refinement were carried out, with three cycles on individual atomic thermal

Neutron diffraction reveals oxygen–histidine hydrogen bond in oxymyoglobin

Simon E. V. Phillips*† & Benno P. Schoenborn†

* MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

† Department of Biology, Brookhaven National Laboratory, Upton, New York 11973, USA

Myoglobin (Mb) reversibly binds molecular oxygen in vertebrate muscle and consists of a polypeptide chain of 153 residues and one haem, which closely resembles one subunit of a haemoglobin (Hb) tetramer. In oxygenated myoglobin (oxyMb) the iron atom is coordinated by four porphyrin nitrogen atoms, N^ε of the invariant 'proximal' histidine (F8), and molecular oxygen¹. The oxygen molecule lies in a tight pocket, bounded by two hydrophobic groups (Phe CD1 Val E11) and the side chain of the 'distal' histidine (E7). This histidine is present in Hb and Mb of many different organisms, with substitution by glutamine or leucine found in only a few cases. The function of the residue is not clear, although it does present steric hindrance to linear ligands such as carbon monoxide and favours 'bent' ones, such as O₂. We report here that the imidazole stabilizes bound molecular oxygen with a hydrogen bond, as revealed by neutron diffraction analysis.

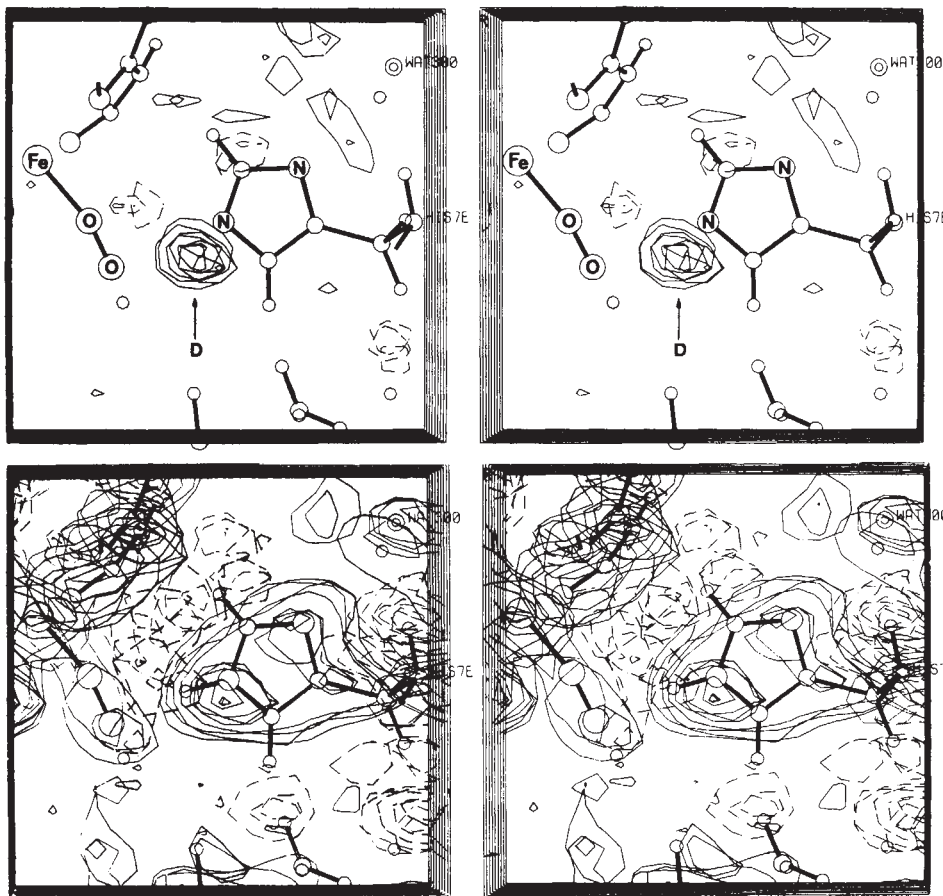


Fig. 2 Stereo view of $|F_o| - |F_c|$ neutron difference density map in a slab 4 Å-thick centred on the plane of E7 imidazole ring. The refined model is superimposed, showing His E7, FeO_2 and part of the haem, in a similar orientation to that in Fig. 1. Contours are $\pm 0.35, 0.55, 0.75$ Fermi per Å, with negative ones shown as broken lines. A strong, positive peak indicates the presence of deuterium bonded to N^ϵ .

Fig. 3 Stereo view of $2|F_o| - |F_c|$ neutron density map in identical orientation to that in Fig. 2. The deuterium has been added to the superimposed model with calculated stereochemistry. The fit to the map is good, although adjustment of the imidazole in later refinement may improve it. Note that whereas D, C, N, O, S and Fe atoms give positive peaks, H scatters out of phase and gives negative peaks. Contours are $-1.0, -0.5, 1.0, 2.0, 3.0, 4.0$ Fermi per Å³.

parameters, reducing R to 18.8%. A difference map calculated at this stage (Fig. 2) shows a strong peak adjacent to N^ϵ of His E7, indicating the N-bound deuterium. An observed density map (coefficients $2|F_o| - |F_c|$) is shown in Fig. 3, and the high concentration of scattering density at N^ϵ compared with N^δ confirms the presence of the D atom. As this atom had not been included in the model for refinement or phase calculation, the peaks result from information present in the observed data alone, and are not biased by assumptions about the structure.

The geometry, summarized in Table 1, indicates a medium strength hydrogen bond, although the coordinates may change a little with further refinement when D is included in the model. Such a bond could contribute several kcal per mol to the enthalpy of oxygen binding, and the position of the E7 imidazole might therefore act as a means of control of oxygen affinity in Hb and Mb.

However, direct observation of the hydrogen bond cannot resolve the controversy between proponents of the Fe(III) -superoxide model for oxygen binding^{9,10}, and those favouring a spin-paired model^{2,11-13} with varying degrees of charge transfer between Fe and O_2 , but it does suggest that molecular orbital calculations attributing no partial negative charge to O-2 should be regarded with caution, as no hydrogen bond was found between N^ϵ and bound CO, where charge transfer is absent¹⁴.

We thank Dr M. F. Perutz for suggesting this experiment and Dr D. F. Koenig for assistance with initial data reduction. This

research was carried out in part at Brookhaven National Laboratory under the auspices of the US Department of Energy, with partial support from the NSF.

Received 16 February; accepted 24 March 1981.

1. Phillips, S. E. V. *J. molec. Biol.* **142**, 531-554 (1980).
2. Pauling, L. *Nature* **203**, 182-183 (1964).
3. Yonetani, T., Yamamoto, H. & Iizuka, T. *J. biol. Chem.* **249**, 2168-2174 (1974).
4. Ikeda-Saito, M., Iizuka, T., Yamamoto, H., Kayne, F. J. & Yonetani, T. *J. biol. Chem.* **252**, 4882-4887 (1977).
5. Ikeda-Saito, M., Brunori, M. & Yonetani, T. *Biochim. biophys. Acta* **533**, 173-180 (1978).
6. Schoenborn, B. P. *Nature* **224**, 143-146 (1969).
7. Norvell, J. C. & Schoenborn, B. P. *Brookhaven Symp. Biol.* **27**, 11-22 (1975).
8. Alberi, J., Fischer, J., Radeka, L. C. & Schoenborn, B. P. *Nucl. Instrum. Meth.* **127**, 507-523 (1975).
9. Weiss, J. J. *Nature* **202**, 83 (1964).
10. Reed, C. A. & Cheung, S. K. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1780-1784 (1977).
11. Olafson, B. D. & Goddard, W. A. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1315-1319 (1977).
12. Case, D. A., Huynh, B. H. & Karplus, M. *J. Am. chem. Soc.* **101**, 4433-4453 (1979).
13. Drago, S. D. & Corden, B. B. *Acc. chem. Res.* **13**, 353-360 (1980).
14. Hanson, J. C. & Schoenborn, B. P. *J. molec. Biol.* (submitted).

Modulation of platelet shape and membrane receptor binding by Ca^{2+} -calmodulin complex

Kuo-Jang Kao, Joachim R. Sommer & Salvatore V. Pizzo

Departments of Pathology and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710, USA

Interaction between factor VIII/von Willebrand factor, a plasma glycoprotein, and platelets is essential for platelet adhesion and subsequent platelet plug formation at sites of vascular injury during primary phase haemostasis^{1,2}. We now report that binding of ¹²⁵I-labelled factor VIII/von Willebrand factor to its platelet membrane receptors is inhibited by preincubation of washed human platelets with inhibitors of Ca^{2+} -calmodulin complex (for example, 50 μM trifluoperazine or

Table 1 Geometry of histidine-oxygen hydrogen bond

	Distance (Å)*		Angle (degrees)
$\text{N}^\epsilon\text{-D}$	1.04	$\text{N}^\epsilon\text{-D} \dots \text{O-2}$	157
$\text{O-2} \dots \text{D}$	1.98	$\text{O-1-O-2} \dots \text{D}$	96
$\text{N}^\epsilon \dots \text{O-2}$	2.97		

Deviation of O-2 from plane of E7 imidazole ring is 0.61 Å.

* Estimated standard deviations for distances, 0.18 Å.

chlorpromazine) at 37 °C for 2 min. Scatchard analysis of the binding data indicates that the total number of accessible binding sites on each pretreated platelet is significantly reduced to ~50% of the original value. There is also negative cooperativity of binding or reduced binding affinity in a major portion of binding sites. Platelet shape changes from diskoid to spherical after treatment. The results suggest that Ca^{2+} -calmodulin complex modulates platelet shape and membrane receptor behaviour.

Recent studies suggest that calmodulin, an intracellular calcium receptor, is important in mediating Ca^{2+} -regulated cellular secretion, motility and enzyme activities (see refs 3, 4 for reviews). Trifluoperazine (TFP) and other phenothiazines are known to bind to Ca^{2+} -calmodulin complexes and block their action⁵. It has been demonstrated^{6,7} in human platelets that calmodulin is essential for the activity of platelet myosin light chain kinase, which is responsible for phosphorylation of myosin light chain and subsequent actomyosin contraction. Moreover, platelet aggregation induced by ADP, thrombin, adrenaline, collagen or factor VIII/von Willebrand factor (FVIII/vWF) is inhibited by inhibitors of Ca^{2+} -calmodulin complex, such as TFP⁸⁻¹⁰. The exact mechanism by which inhibition of Ca^{2+} -calmodulin complex function results in failure of platelet aggregation is unknown. Recently, Gerrard *et al.*¹¹ reported that platelet membrane glycoprotein III immunologically cross-reacts with α -actinin and may anchor actin filaments. Phillips *et al.*¹² reported a close association between actomyosin filaments and platelet membrane glycoproteins. In view of these findings and regulation of distribution of membrane proteins by cytoskeletal elements in other cellular systems¹³, we have examined the possibility that inhibitors of Ca^{2+} -calmodulin complex inhibit the binding of platelet aggregating agents to their membrane receptors. As we have previously demonstrated that binding of FVIII/vWF to its platelet receptors is responsible for FVIII/vWF-induced platelet aggregation¹⁴, the effects of the Ca^{2+} -calmodulin inhibitors, TFP and chlorpromazine (CP), on platelet shape, and the binding of FVIII/vWF to its platelet membrane receptors were studied.

Human platelets used in these experiments were prepared from citrated venous blood of healthy donors who had received no drugs for at least 2 weeks before the study. Fresh platelets were collected by differential centrifugation¹⁵ and washed first with Tyrode's buffer, pH 7.4, containing HEPES (2 mM), EDTA (5 mM), apyrase (75 $\mu\text{g ml}^{-1}$) but no Ca^{2+} or Mg^{2+} . The platelets were washed again in the same buffer (without apyrase) and resuspended in Tyrode's buffer, pH 7.4, containing 2 mM HEPES. The concentration of washed platelets was determined

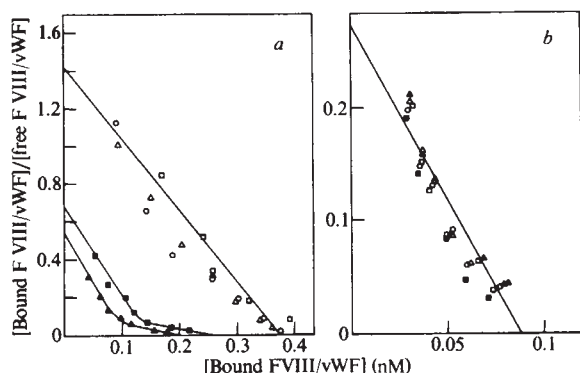


Fig. 1 Scatchard analysis of ^{125}I -FVIII/vWF binding to platelets pretreated with different phenothiazines. The binding data used for the analysis were obtained by incubating platelets with increasing concentrations of ^{125}I -FVIII/vWF in the presence of ristocetin (1 mg ml^{-1}) as described elsewhere¹⁴. In *a*, washed fresh platelets (5×10^6 per incubation) were used. In *b*, paraformaldehyde-fixed platelets (1.2×10^6 per incubation) were used. $\circ$, Control; $\blacktriangle$, TFP pretreated; $\triangle$, TFP sulphoxide pretreated; $\blacksquare$, CP pretreated; $\square$, CP sulphoxide pretreated.

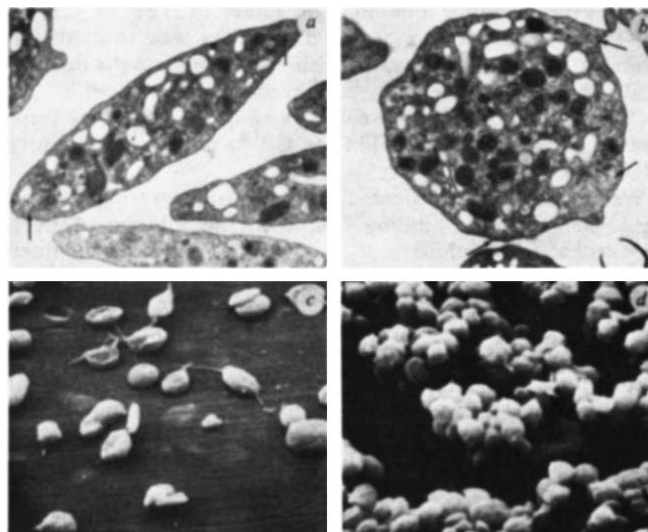


Fig. 2 Transmission electron micrographs of platelets pretreated with TFP; *a* shows the control platelets ($\times 24,500$). The platelet is diskoid shaped. Electron-dense granules, α granules, and the open canalicular system are visible. Cross-sections of peripheral circular microtubules (arrows) are visible at both ends of the platelet. Similar findings are observed in platelets pretreated with DMSO (0.5%) or TFP sulphoxide (50 μM). *b*, Platelets pretreated with TFP (50 μM) at 37 °C for 5 min ($\times 24,500$); the platelets have become spheroid in shape. Redistribution of microtubules is indicated by arrows. The cross-sections of peripheral microtubules (arrows) suggest that the platelet in *b* has the same orientation as that in *a*. The change in shape of platelets after TFP treatment was confirmed by scanning electron microscopy (*c*, *d*). *c*, Control platelets which appear as disks ($\times 33,000$). Similar morphology was observed in DMSO (0.5%) or TFP sulphoxide (50 μM)-treated platelets. *d*, TFP (50 μM)-treated platelets (37 °C, 2.5 min). The platelets appear as spheres ($\times 3,000$). Platelets were fixed as described in the text and prepared for scanning electron microscopy as described elsewhere²¹. Platelets were sputter-coated with platinum and examined using an SEM-Phillips 501.

in a haemocytometer by phase-contrast microscopy. The washed platelets were used for all studies of platelet aggregation, FVIII/vWF receptor binding and shape changes. The binding studies were carried out as described in detail elsewhere¹⁴.

To demonstrate that the washed platelets were still responsive to treatment with Ca^{2+} -calmodulin inhibitors, the effect of TFP or CP on platelet aggregation induced by FVIII/vWF or thrombin was studied by turbidimetry¹⁴ in a Chronolog model-440 aggregometer at 37 °C. The washed platelets (5×10^7 platelets ml^{-1}) were aggregated by thrombin (0.5 U ml^{-1}) or by FVIII/vWF (2 $\mu\text{g ml}^{-1}$) in the presence of ristocetin (1 mg ml^{-1}). Platelet aggregation was markedly inhibited by either TFP or CP (50 μM each) but not by the same concentrations of their inactive sulphoxide derivatives (TFP sulphoxide or CP sulphoxide). This result demonstrates not only that platelets are responsive to TFP or CP after our washing procedures, but also confirms the notion that platelet Ca^{2+} -calmodulin complex plays an essential part in platelet aggregation induced by FVIII/vWF, thrombin or other agents⁸⁻¹⁰.

The effect of TFP or CP on ^{125}I -labelled FVIII/vWF binding to platelet receptors was then studied. The specific binding of ^{125}I -FVIII/vWF to platelets was markedly inhibited when the washed platelets were preincubated with TFP (50 μM) at 37 °C for 2 min (Table 1). No inhibition of receptor binding was observed when platelets were preincubated with TFP sulphoxide (50 μM), CP sulphoxide (50 μM) or dimethyl sulphoxide (DMSO: 0.5%, v/v). Recently, we have demonstrated that platelet membrane FVIII/vWF receptors can be fully preserved after fixation of platelets with 2% paraformaldehyde¹⁴. To exclude the possible direct interfering effect of TFP or CP on

FVIII/vWF receptor binding, the effect of TFP or CP on ^{125}I -FVIII/vWF binding to fixed platelets was studied. No inhibitory effect was observed (Table 1). These results demonstrate that neither TFP nor CP directly interferes with ^{125}I -FVIII/vWF binding to the receptors and that responsive fresh platelets are required for TFP or CP to have any inhibitory effect.

We also studied the effect of different concentrations of TFP on ^{125}I -FVIII/vWF binding (Table 2); an inhibitory effect, apparent after the addition of 25 μM of TFP, reached a plateau after addition of 100 μM of TFP. This dose response is consistent with the observations of White and Raynor¹⁰ who studied the effect of TFP concentrations on thrombin-induced platelet aggregation and serotonin release. The fact that TFP inhibition of platelet-aggregating agents thrombin and FVIII/vWF is concentration dependent suggests that TFP may inhibit platelet function by acting on Ca^{2+} -calmodulin complex.

Kinetic studies were made to determine whether the inhibitory effect of TFP or CP on receptor binding results from reduced receptor binding affinity or a decreased number of accessible binding sites on each platelet. Scatchard analysis of the binding data revealed that the total number of accessible binding sites on each platelet was significantly reduced after platelets were preincubated with TFP or CP (Fig. 1a). Curvilinear Scatchard plots were also obtained. These results indicate either that there is negative cooperativity of binding or that a portion of binding sites shift towards low-affinity binding after pretreatment of platelets with inhibitors of Ca^{2+} -calmodulin complex. Again, these effects were not observed in the fixed platelets (Fig. 1b). Our observations suggest that treatment of platelets with Ca^{2+} -calmodulin inhibitors may affect the distribution or the aggregation state of the receptor in the plasma membrane. Thus the accessibility of the receptors for the ligand or the receptor binding behaviour is affected.

We therefore studied the ultrastructural changes of platelets pretreated with TFP or CP using transmission electron microscopy and standard freeze-fracture techniques. Washed platelets were pretreated with different derivatives of the phenothiazines (50 μM) or DMSO (0.5%, v/v) at 37 °C for different time periods. The treated platelets were fixed by adding an equal volume of 1.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4, containing 5% sucrose, for 1 h at room temperature. The fixed platelets were sedimented and processed for freeze-fracture using a method described elsewhere¹⁶. Fixed platelets were

Table 2 Effect of different concentrations of trifluoperazine on ^{125}I -FVIII/vWF binding to washed fresh platelets

	TFP (μM)					
	0	10	25	50	100	200
Specific binding (c.p.m.)	47,911	48,875	47,111	40,317	20,907	19,400
% of control	100	102.0	98.3	84.1	43.6	40.5

Specific binding was determined as described in Table 1 legend. The specific radioactivity of ^{125}I -FVIII/vWF was 9.5×10^5 c.p.m. per μg .

also sedimented, fixed by 2% osmium tetroxide, dehydrated and embedded in Epon. Thin sections of sample were cut, stained with uranyl acetate and lead acetate, then examined using a Zeiss EM-10 electron microscope. Results of the study showed that distribution of membrane particles in both the E and P face of the platelet plasma membrane was not affected by pretreatment with TFP (50 μM) or CP (50 μM) at 37 °C for different time periods (2–45 min, data not shown). Nevertheless, thin sections of platelets showed the redistribution of microtubules and that platelets changed from their original diskoid shape into spheres after pretreatment with TFP or CP at 37 °C for 20 min (Fig. 2a, b). This finding was further confirmed by examination of platelets with a scanning electron microscope (Fig. 2c, d). This change in platelet shape was not observed after pretreatment with TFP sulphoxide or CP sulphoxide. Inhibitors of Ca^{2+} -calmodulin may effect the change in platelet shape by inhibiting the normal function of actomyosin and microtubules, as it is known that both these cytoskeletal elements are essential for maintaining platelet shape^{11,17} and the Ca^{2+} -calmodulin complex regulates microtubule assembly/disassembly and platelet myosin light-chain kinase^{6,7}. In addition, we observed that platelets became spherical after pretreatment with 25 μM TFP at 37 °C for 2 min. The degree of change in shape was not significantly different between platelets treated with 25 μM and 50 μM TFP. However, there was a significant difference in inhibition of ^{125}I -FVIII/vWF binding (Table 2). Thus, it is unlikely that the reduced binding of FVIII/vWF resulted from the change in platelet shape.

Our results are substantiated by recent observations that intra-platelet cyclic AMP inhibits FVIII/vWF binding to platelets^{18,19}. It is known that cyclic AMP acts as a Ca^{2+} antagonist by enhancing Ca^{2+} uptake into the platelet Ca^{2+} storage sites in the dense tubular system²⁰. The results of this study demonstrate that Ca^{2+} -calmodulin complex may have an important role in regulating platelet membrane receptor behaviour and maintaining the diskoid shape of platelets in the resting state.

This work was supported by US PHS grants HL 24066, HL 15448 and HL 12486 and the Veterans Administration Research Service.

Received 10 April; accepted 21 April 1981.

1. Sakariassen, K. J., Bolhuis, P. A. & Sixma, J. J. *Nature* **279**, 636–638 (1979).
2. Hovig, T. & Stormakem, H. *Acta path. microbiol. scand.* **248**, 105–122 (1974).
3. Means, A. R. & Dedman, J. R. *Nature* **285**, 73–77 (1980).
4. Cheung, W. Y. *Science* **207**, 19–27 (1980).
5. Weiss, B., Prozialek, W., Cimino, M., Barnette, M. S. & Wallace, T. L. *Ann. N.Y. Acad. Sci.* **356**, 319–345 (1980).
6. Hathway, D. R. & Adelstein, R. S. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1653–1657 (1979).
7. Adelstein, R. S., Conti, M. A. & Pato, M. D. *Ann. N.Y. Acad. Sci.* **356**, 142–150 (1980).
8. Kindness, G., Williamson, F. B. & Long, W. F. *Thromb. Res.* **17**, 549–554 (1980).
9. Kruckberg, W. C., Lowery, M. S. & Brewer, G. J. *Clin. Res.* **27**, 744A (1979).
10. White, G. C. & Raynor, S. T. *Thromb. Res.* **18**, 279–284 (1980).
11. Gerrard, J. M., Schollmeyer, J. V., Phillips, D. R. & White, J. G. *Am. J. Path.* **94**, 509–528 (1979).
12. Phillips, D. R., Jennings, L. K. & Edwards, H. H. *J. Cell Biol.* **86**, 77–86 (1980).
13. Korn, E. D. in *Cell Motility. Cold Spring Harb. Conf. Vol. 3B* (eds Goldman, R., Pollard, T. & Rosenbaum, J.) 623 (Cold Spring Harbor Laboratory, New York, 1976).
14. Kao, K. J., Pizzo, S. V. & McKee, P. A. *J. clin. Invest.* **63**, 656–664 (1979); *Proc. natn. Acad. Sci. U.S.A.* **76**, 5317–5320 (1979); *Blood* **57**, 579–585 (1981).
15. Kinlough-Rathbone, R. L. *et al. Thromb. Haemostasis* **37**, 291–308 (1977).
16. Sommer, J. R., Wallace, N. R. & Junker, J. *J. ultrastruct. Res.* **71**, 126–142 (1980).
17. Barnhart, M. I. *Molec. cell. Biochem.* **22**, 113–137 (1978).
18. Tang, S. S., Moake, J. L., Trolli, J. H. & Olson, J. D. *Clin. Res.* **28**, 325A (1980).
19. Collier, B. S. *Clin. Res.* **28**, 307A (1980).
20. Kaser-Glanzmann, E., Gerber, E. & Luscher, E. F. *Biochim. biophys. Acta* **558**, 344–347 (1979).
21. White, J. G. & Krumwiede, M. *Blood* **41**, 823–832 (1973).

Table 1 Effect of trifluoperazine and chlorpromazine on ^{125}I -FVIII/vWF binding to washed fresh platelets and paraformaldehyde-fixed platelets

Pretreatment	Specific binding (c.p.m.)	
	Washed fresh platelets	Paraformaldehyde-fixed platelets
Control	37,625 (100.0)	38,624 (100.0)
DMSO	36,272 (96.4)	38,243 (99.0)
TFP	17,902 (46.3)	38,204 (98.9)
TFP sulphoxide	36,068 (95.9)	37,434 (96.9)
CP	28,594 (75.9)	36,052 (93.3)
CP sulphoxide	36,220 (96.3)	37,140 (96.2)

The specific binding of ^{125}I -FVIII/vWF to 5×10^6 platelets was determined as described elsewhere¹⁴. The specific radioactivity of the ^{125}I -FVIII/vWF protein was 7.3×10^5 c.p.m. per μg . TFP, CP, TFP sulphoxide and CP sulphoxide were dissolved in DMSO. The platelet suspensions were preincubated with TFP, CP, TFP sulphoxide or CP sulphoxide at 37 °C for 2 min; the final concentrations of all these reagents was 50 μM . The final concentration of DMSO was 0.5% (v/v). All studies were carried out in duplicate; variation between duplicates was <5%. The experiments were repeated three times, with similar results each time. Only one representative experiment is shown here. TFP sulphoxide and CP sulphoxide are derivatives which inhibit Ca^{2+} -calmodulin complex to an extent which is only 2–3% of that of TFP or CP⁵. It is unknown whether these derivatives enter platelets. % Binding of control is given in parentheses.

Organic solvents modify the calcium control of flagellar movement in sea urchin sperm

Barbara H. Gibbons* & I. R. Gibbons

Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii 96822, USA

The form of flagellar and ciliary beating in various organisms is regulated by the intracellular concentration of free Ca^{2+} (refs 1–5). In demembrated sea urchin sperm reactivated with 1 mM Mg-ATP^{2-} , Ca^{2+} regulates the asymmetry of the flagellar waveform^{6–8}. On reactivation at a free Ca^{2+} concentration below 10^{-8} M, the flagella beat with almost symmetric bending waves⁹, and the sperm swim in nearly straight paths with an average turning rate of ~ 0.04 radians per beat, whereas in the presence of 10^{-3} M free Ca^{2+} , the bending waves are highly asymmetric and the sperm describe circular paths with an average turning rate between 0.2–0.35 radians per beat. Here we show that low concentrations of various organic solvents interact with the mechanism by which Ca^{2+} induces waveform asymmetry. The solvents studied fall into two groups of which the first, consisting of methanol, 2-propanol and ethylene glycol, mimic the effect of Ca^{2+} and increase the asymmetry, whereas the second group, N,N' -dimethylformamide, formamide and p -dioxane, block the increase in asymmetry induced by Ca^{2+} . However, all solvents in both groups act similarly in decreasing the flagellar beat frequency.

The effects of methanol and dimethylformamide on the asymmetry of reactivated sperm in the presence or absence of added Ca^{2+} are shown in Fig. 1. Methanol increases the asymmetry in both cases. On the other hand, dimethylformamide decreases the asymmetry induced by Ca^{2+} and has almost no effect on the slight asymmetry found in the absence of added Ca^{2+} . The decrease in flagellar beat frequency induced by these two solvents is shown in Fig. 2. The preparations of sperm remain almost 100% motile until sufficient solvent has been added to lower the frequency from ~ 28 Hz to 18 Hz, beyond which the numbers of non-motile sperm increase. Table 1 shows the relative sensitivity of the sperm to the various solvents tested. With most of the solvents, the relative concentrations needed to produce the chosen standard effects on asymmetry and beat frequency were about the same; however, the action of formamide seems to be relatively selective for asymmetry.

The changes in symmetry and beat frequency induced by the solvents seem to be complete within the time of mixing and to remain constant for ~ 5 min. The effects of methanol and 2-propanol seem to be fully reversible if the sperm are diluted 10-fold out of the solvent solution into fresh reactivating solution within ~ 5 min. The blockage of Ca^{2+} -induced asymmetry by dimethylformamide and dioxane, however, is only partially reversed on dilution of the sperm into fresh reactivating solution containing 1 mM free Ca^{2+} but no solvent, although the beat frequency immediately returns to its original value. In the average of four preparations, the initial turning rate of 0.32 radians per beat fell to 0.11 radians per beat on addition of 0.9 mol % dimethylformamide and then rose to only 0.17 radians per beat after 50-fold dilution of the solvent. However, the asymmetric beating of such preparations could be fully restored by the addition of 3.2 mol % methanol, or 1.7 mol % methanol and 0.1 mM free Ca^{2+} .

Brief digestion with trypsin desensitizes reactivated sperm flagella to Ca^{2+} , so that the flagellar bending waves become nearly symmetric even in the presence of millimolar concentrations of free Ca^{2+} (refs 10, 11). This suggests that brief trypsin

digestion destroys one or more structures that mediate the Ca^{2+} regulation of asymmetry, while leaving intact the ability of the flagella to propagate symmetric bending waves. We have compared the effect of mild trypsin digestion on the movement of reactivated sperm induced to beat asymmetrically by addition of 3.9 mol % methanol (in the absence of added Ca^{2+}) with its effect on a control sample containing reactivated sperm induced to beat asymmetrically by the addition of 1 mM free Ca^{2+} but no methanol. After addition of 0.2 μg trypsin per ml of reactivating

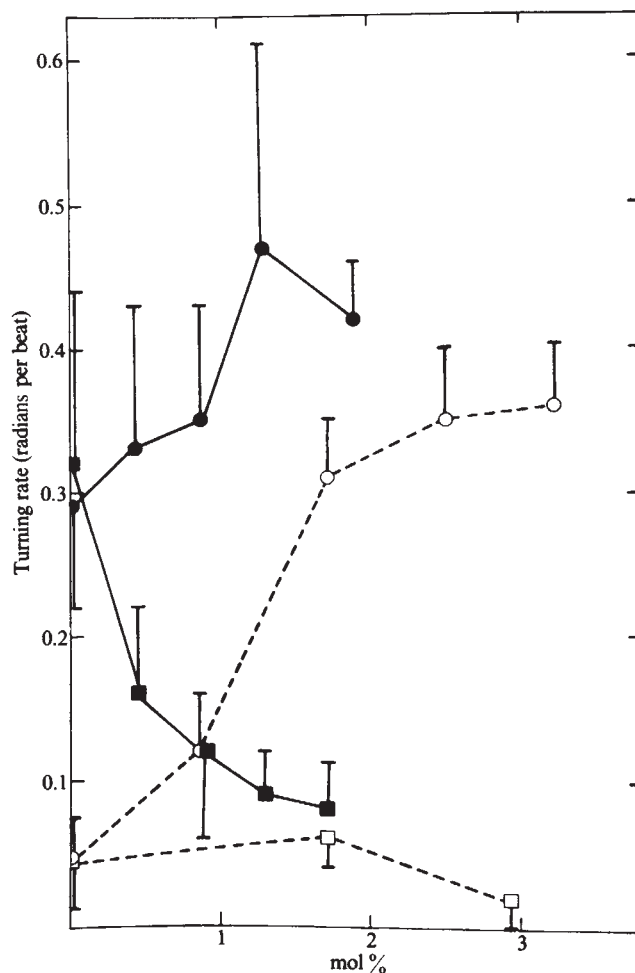


Fig. 1 Effect of solvent concentration on turning rate of demembrated sperm reactivated at 22–23 °C with 1 mM Mg-ATP^{2-} in buffered solution containing either 0.1 mM EGTA (open symbols) or 0.1 mM EGTA and added Ca^{2+} (1.2 mM) (solid symbols). In these two conditions, the actual concentrations of Ca^{2+} not complexed to EGTA or ATP are estimated to be $<10^{-8}$ M and ~ 1 mM, respectively²³. Procedures for demembrating and reactivating sperm of the sea urchin, *Tripneustes gratilla*, were as described earlier for 'potentially symmetric sperm' (ref 8). ●, Methanol and free Ca^{2+} (~ 1 mM); ○, methanol and free Ca^{2+} ($<10^{-8}$ M); ■, N,N' -dimethylformamide and free Ca^{2+} (~ 1 mM); □, N,N' -dimethylformamide and free Ca^{2+} ($<10^{-8}$ M). Solvents were Glass Distilled grade from Burdick and Jackson, Inc., Muskegon, Michigan; equivalent results were obtained with highly purified grades from other sources. The solvents were diluted 1:1 with reactivating solution to minimize the heat of solution before addition to the sperm. Concentrations are given as mol % (mol fraction $\times 100$). For comparison with other units, 1.0 mol % equals 2.2% (v/v) or 0.55 M methanol, and 3.3% (v/v) or 0.53 M dimethylformamide. Turning rates were determined from time exposures taken at a magnification of $\times 160$ on Polaroid 3000 Type 107C film by measuring the angle of circular arc travelled by the sperm head during an exposure of known duration. This value combined with the flagellar beat frequency, determined using a stroboscopic flash unit, gave the turning rate in radians per beat. Each point represents the mean obtained from measurements of 10–30 sperm, and the error bars indicate the standard deviation.

* To whom correspondence should be addressed at Kewalo Marine Laboratory, University of Hawaii, 41 Ahui Street, Honolulu, Hawaii 96813, USA.

Table 1 Relative effectiveness of solvents

Concentration required to induce asymmetric beating* (mol %)		Concentration required to block Ca^{2+} -induced asymmetric beating† (mol %)		Concentration required to lower beat frequency‡ (mol %)	
Ethylene glycol	4.5	<i>p</i> -Dioxane	0.7	Ethylene glycol	4.6
Methanol	2.5	<i>N,N'</i> -Dimethylformamide	0.6	Methanol	3.2
2-Propanol	0.5	Formamide	0.4	Formamide	2.5
				<i>N,N'</i> -Dimethylformamide	1.5
				2-Propanol	0.6
				<i>p</i> -Dioxane	0.6

* The concentration (mol %) needed to raise turning rate to ~ 0.34 radians per beat in sperm reactivated with 1 mM ATP at 22–23 °C in a buffer containing 10 mM Tris-HCl, pH 8.1, 0.15 M KCl, 2 mM MgSO_4 , 0.1 mM EGTA, 1 mM dithiothreitol and 2% (w/v) polyethylene glycol (molecular weight 20,000).

† The concentration (mol %) needed to reduce turning rate to half its initial value in sperm reactivated with 1 mM ATP at 22–23 °C in the same buffer as above, but with CaCl_2 added (1.1 mM).

‡ The concentration (mol %) needed to reduce beat frequency by 9 Hz, with conditions as in first column for ethylene glycol, methanol and 2-propanol, and conditions as in second column for *p*-dioxane, dimethylformamide and formamide.

solution the waveforms became gradually more symmetric at about the same rate in both samples, until after 2–3 min the sperm swam in almost straight lines with apparently normal, symmetrical waveforms. The parallel elimination of both Ca^{2+} -induced and methanol-induced asymmetric bending waves by trypsin digestion suggests that these two agents share, at least in part, a common pathway in regulating asymmetry, and that the trypsin-sensitive structure is located in this shared portion.

While the effects of the solvents on asymmetry divide them into two opposing groups, their effect on beat frequency is in the same direction in all cases and may be due to a direct alteration of the kinetic properties of dynein ATPase¹². This second action of the solvents seems to be distinct from their action on asymmetry as it is unaffected by trypsin digestion, and it is also distinct from that of Ca^{2+} , which, at up to 2 mM in the conditions used here, has only a small effect on beat frequency⁷.

The relative effectiveness of the solvents in altering the asymmetry of flagellar waveforms seems to be unrelated to their dielectric constants, which for solvents of the second group range from ~ 2 for dioxane to 109 for formamide¹³, while their relative effectiveness on asymmetry varies less than twofold.

Cilia and flagella contain calmodulin-like proteins that presumably act as Ca^{2+} -receptors^{14–17}. Binding of Ca^{2+} to

bovine brain calmodulin induces the exposure of a hydrophobic surface on the protein which then becomes capable of interaction with various hydrophobic ligands in a manner that may mimic its interaction with the phosphodiesterase molecule *in vivo*¹⁸. Organic solvents generally induce unfolding of protein structure by promoting the transfer of hydrophobic side chains from internal nonpolar domains of the native structure to the external organic solvent/water solution which provides a less polar environment than water alone¹⁹. Our results could be explained by assuming that methanol and 2-propanol themselves induce the exposure of the hydrophobic surface in a way similar to the action of Ca^{2+} , while dimethylformamide and dioxane (in the presence of elevated Ca^{2+}) competitively inhibit the binding of this domain to its subsequent substrate. However, a common feature of the solvents of the second group is the presence of relatively polar, oxygen-containing moieties that could act as hydrogen acceptors and thus interact with proteins by competing for hydrogen bonds, and this provides a possible alternative mechanism for the action of these solvents²⁰. In any case, the fact that the effects described here were induced by concentrations of solvent that are low relative to those frequently used in studies of protein unfolding^{21,22}, suggests that these solvents have a specific effect on sensitive regulatory protein subunits. Thus, it may be profitable to investigate the effects of these two groups of solvents on other Ca^{2+} -regulated systems as well as on the conformation of the solubilized Ca^{2+} -receptor proteins.

This work was supported by NIH grant HD 06565.

Received 12 January; accepted 9 April 1981.

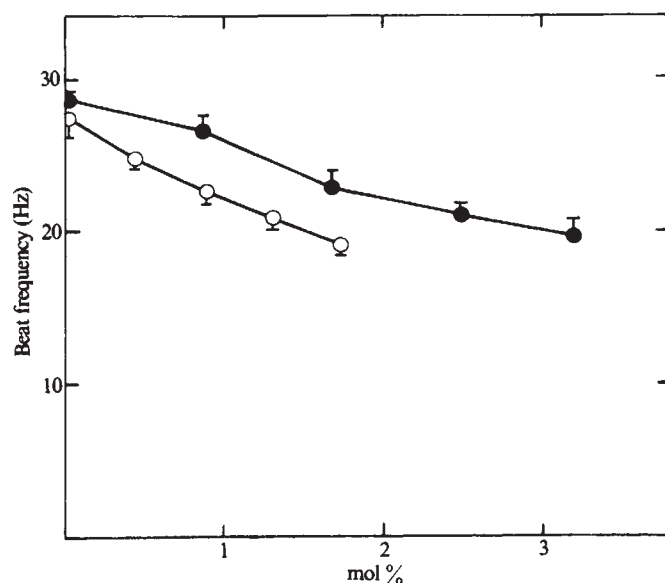


Fig. 2 Flagellar beat frequency of sperm reactivated with 1 mM Mg-ATP^{2-} at 22–23 °C as a function of added solvent: ●, methanol and free Ca^{2+} ($<10^{-8}$ M); ○, dimethylformamide and free Ca^{2+} (~ 1 mM). Conditions are as described in Fig. 1 legend. Each point represents the mean ($\pm$ s.d.) obtained from measurements of 8–22 sperm.

- Naitoh, Y. & Kaneko, H. *Science* **176**, 523–524 (1972).
- Murakami, A. & Takahashi, K. *J. Fac. Sci. Tokyo Univ.* **13**, 251–256 (1975).
- Holwill, M. E. J. & McGregor, J. L. *J. exp. Biol.* **65**, 229–242 (1976).
- Hyams, J. S. & Borisy, G. G. *J. Cell Sci.* **33**, 235–253 (1978).
- Gibbons, B. H. *J. Cell Biol.* **84**, 1–12 (1980).
- Brokaw, C. J., Josslin, R. & Bobrow, L. *Biochem. biophys. Res. Commun.* **58**, 795–800 (1974).
- Brokaw, C. J. *J. Cell Biol.* **82**, 401–411 (1979).
- Gibbons, B. H. & Gibbons, I. R. *J. Cell Biol.* **84**, 13–27 (1980).
- Gibbons, B. H. & Gibbons, I. R. *J. Cell Biol.* **54**, 75–97 (1972).
- Brokaw, C. J. & Gibbons, I. R. in *Swimming and Flying in Nature* Vol. 1 (eds Wu, T. Y. -T., Brokaw, C. J. & Brennan, C.) 89–126 (Plenum, New York, 1975).
- Brokaw, C. J. & Simonick, T. F. *J. Cell Biol.* **75**, 650–665 (1977).
- Holwill, M. E. J. *J. exp. Biol.* **50**, 203–222 (1969).
- Riddick, J. A. & Bunger, W. B. in *Techniques of Chemistry: Organic Solvents. Physical Properties and Methods of Purification* Vol. 2, 3rd edn (Wiley-Interscience, New York, 1970).
- Jamieson, G. A. Jr, Vanaman, T. C. & Blum, J. J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6471–6475 (1979).
- Van Eldik, L. J., Piperno, G. & Watterson, D. M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4779–4783 (1980).
- Garbers, D. L., Hansbrough, J. R., Radany, E. W., Hyne, R. V. & Kopf, G. S. *J. Reprod. Fert.* **59**, 377–381 (1980).
- Gitelman, S. E. & Witman, G. B. *J. Cell Biol.* **87**, 764–770 (1980).
- LaPorte, D. C., Wierman, B. M. & Storm, D. R. *Biochemistry* **19**, 3814–3819 (1980).
- Tanford, C. in *The Hydrophobic Effect* 2nd edn (Wiley, New York, 1980).
- Jencks, W. P. in *Catalysis in Chemistry and Enzymology*, 332–336 (McGraw-Hill, New York, 1969).
- Herskovits, T. T., Gadegbeku, B. & Jallat, H. *J. biol. Chem.* **245**, 2588–2598 (1970).
- Parodi, R. M., Bianchi, E., & Ciferri, A. *J. biol. Chem.* **248**, 4047–4051 (1973).
- Caldwell, P. C. in *Calcium and Cellular Function* (ed. Cuthbert, A. W.) 10–16 St Martin's, New York, 1970).

Co-translational membrane integration of calcium pump protein without signal sequence cleavage

Keith E. Mostov*, Paul DeFoor†, Sidney Fleischer† & Günter Blobel*

* Laboratory of Cell Biology, The Rockefeller University, New York, New York 10021, USA

† Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235, USA

The calcium pump protein (CPP) or Ca^{2+} -ATPase is the predominant integral membrane protein of the sarcoplasmic reticulum (SR) of skeletal muscle^{1,2}. CPP is a single polypeptide chain of molecular weight (MW) 119,000 (ref. 3) which loops in and out of the membrane at least three times⁴⁻⁸. Recently, a number of integral transmembrane proteins possessing a single hydrophilic domain on each side of the membrane have been observed to use a signal sequence, cleaved^{9,10} or uncleaved¹¹, to initiate translocation of their ectoplasmic domain¹². Microsomal membranes when present during translation are capable of correctly integrating these *de novo* synthesized proteins into the membrane^{9,11}. We have used this *in vitro* translocation system to investigate the integration of CPP into the microsomal membrane. We found that CPP is synthesized without a cleaved signal sequence and that it can be integrated into heterologous microsomal membranes only when these are present during translation, but not when they are present after completion of translation.

Translation of mRNA from muscles of 1-day-old rabbits in the wheat-germ cell-free system (in the absence of pancreas microsomal vesicles), followed by immunoprecipitation of the total translation products with sheep anti-rabbit CPP serum yielded a single product (Fig. 1, lane 2) of an apparent molecular weight identical to that of authentic CPP (lane 1).

It has recently been established that CPP translated in the reticulocyte lysate system in the absence of microsomal membranes retains the initiator methionine¹³. However, this experiment did not indicate whether CPP was synthesized with a signal peptide which would be proteolytically removed by the microsomal membranes. To investigate this directly, we performed a partial amino-terminal sequence analysis of CPP synthesized *in vitro* in the absence of microsomal membranes (Fig. 2) and found substantial agreement with the known sequence of CPP from the SR¹⁴ (Met in position 1; Ala in 3, 4 and 14; Cys in 12). It can therefore be concluded that CPP is synthesized without a cleaved amino-terminal signal sequence. This conclusion is firmly based on previous data which demonstrate that the wheat germ cell-free system *per se*, that is, not supplemented by microsomal membranes, is free of that signal peptidase activity which cleaves signal sequences addressed to the endoplasmic reticulum. This signal peptidase activity is introduced into the wheat-germ cell-free system only on supplementation of microsomal membranes^{9,15}.

From cell fractionation of muscle and subsequent incubation of cell fractions containing free or membrane-bound polysomes in a protein synthesizing system, it has been established that CPP is synthesized by the membrane-bound polysome fraction^{16,17}. Based on the criterion of resistance to extraction by low concentrations of detergent¹⁷, the newly synthesized CPP was integrated into the homologous microsomal membranes.

To investigate whether *de novo* synthesized CPP can be integrated into heterologous (dog pancreas) microsomal membrane vesicles, and if so, whether integration is coupled to translation, we carried out experiments in which microsomal vesicles were present either at the start or after completion of translation.

To assay for integration we had hoped to use protection from limited trypsin digestion as a criterion because tryptic digestion of CPP in SR vesicles is known to yield several discrete fragments that are resistant to further degradation, presumably because of their being embedded in the lipid bilayer⁴⁻⁸. However, we found that the generation of these characteristic tryptic fragments was not the result of integration of CPP into the lipid bilayer. Almost identical fragments were produced when trypsin digestion of authentic CPP was carried out in the absence (Fig. 3, lanes 5-7) or presence (Fig. 3, lanes 2-4) of the nonionic detergent Triton X-100. When the *in vitro* product was digested with trypsin in a wide variety of conditions, these fragments were never found; regardless of whether the CPP was translated in the presence or absence of microsomal vesicles, only much smaller tryptic fragments were observed (data not shown). This may indicate that the *in vitro* product never became integrated into the microsomal membranes or that it was

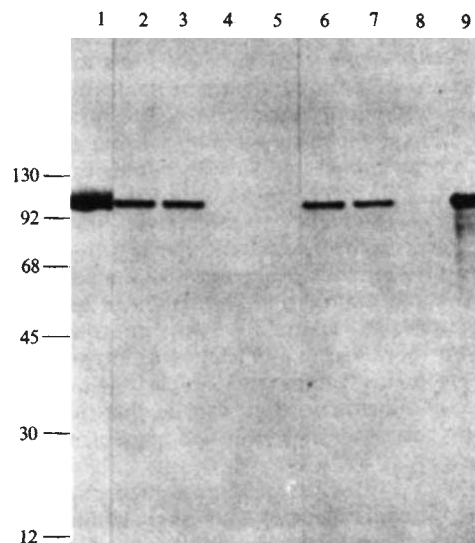


Fig. 1 Cell-free translation and membrane integration of CPP. Lane 1 is from a Coomassie blue-stained gel of authentic CPP². Lanes 2-9 are from a fluorographed gel of *in vitro* translated products. Total mRNA was isolated from the limb and back muscles of 1-day-old rabbits¹¹ and translated in the wheat-germ cell-free translation system with ³⁵S-methionine¹¹. Sheep anti-rabbit CPP serum²⁸ and protein A-Sepharose were used to immunoprecipitate the translated CPP, and the immunoprecipitates were analysed on 7.5-15% polyacrylamide SDS gels and detected by fluorography. Lane 2 is an immunoprecipitate of a 20-μl translation. The specificity of the immunoprecipitation is indicated by the ability of excess (25 μg) authentic CPP to block the immunoprecipitation (lane 4) and the inability of normal sheep serum alone to immunoprecipitate the product (lane 5). Lane 3 is from a 50-μl reaction performed in the presence of microsomal vesicles from dog pancreas²⁹. Note that the mobility of the *in vitro* product is not changed. In lane 6, a 100-μl translation performed in the presence of dog pancreas microsomes was incubated for 180 min at 25 °C, instead of the normal 90 min. After translation the pH was adjusted to 11.5 by adding 5 μl of 1 M NaOH. After 15 min at 0 °C, the 105 μl was layered over a sucrose gradient in a Beckman airfuge tube which consisted of 80 μl of 0.2 M sucrose and 20 μl of 2 M sucrose. The sucrose solutions contained the same salts as the translation mixture and were also adjusted to pH 11.5 with NaOH. The gradients were centrifuged at 160,000 g for 15 min, then 120 μl were removed from the top (supernatant) and the microsomes collected from the 0.2 m/2 M interface and diluted to 120 μl with a solution which contained the same salts as the translation mixture, also adjusted to pH 11.5. Both samples were adjusted to 2% SDS, heated to 100 °C for 5 min, then neutralized and immunoprecipitated. Lane 6 is the immunoprecipitate of the microsomes while lane 7 is that of supernatant. To demonstrate that integration does not occur post-translationally, the same experiment was performed, except that microsomal vesicles were added only after the translation had been incubated for 90 min at 25 °C (at which point no further incorporation of ³⁵S-methionine into protein was detectable) and the incubation continued for a further 90 min at 25 °C. Lane 8 is the immunoprecipitate of the microsomes; lane 9 is that of the supernatant. As a positive control, we demonstrated that an integral membrane protein (the G protein of vesicular stomatitis virus) which is co-translationally but not post-translationally integrated into the dog pancreas microsomes⁹, will resist extraction in these conditions only when the protein is properly integrated into the heterologous microsomes (unpublished data). The positions of the molecular weight markers (in thousands) are indicated. Proteins used, in order of decreasing size were: *Escherichia coli* β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase and cytochrome c.

integrated but did not assume the native conformation found in the SR. Alternatively, the characteristic tryptic fragments of authentic CPP may be the result of intermolecular association, because CPP exists as an oligomer, both in the membrane^{18,19} and in detergent solution²⁰. The *in vitro* CPP may be present in too low a concentration to form oligomers which might confer partial resistance to tryptic digestion.

As an alternative assay for membrane integration, we determined whether CPP could be extracted by alkaline solution (pH 11.5), as integral membrane proteins are non-extractable²¹⁻²³. By this criterion, about half of the *de novo* synthesized CPP molecules were integrated into dog pancreas microsomal vesicles, but only when these were present during translation (Fig. 1, lanes 6, 7)—not when they were added after translation (Fig. 1, lanes 8, 9). It remains to be shown, however, whether the observed integration into the dog pancreas microsomal vesicles was normal, that is, whether the folding of the polypeptide backbone in these vesicles is identical to that of its *in vivo* counterpart in muscle rough endoplasmic reticulum.

The observed coupling between synthesis of CPP and its integration into heterologous microsomal membranes are analogous to those observed previously for translocation of secretory proteins and integration of bitopic integral membrane proteins, suggesting a similar membrane-catalysed mechanism^{12,24}. The recent purification of endoplasmic reticulum-associated proteins²⁵ that catalyse translocation of secretory proteins across the endoplasmic reticulum membrane and the demonstration that these same proteins also catalyse the integration into the endoplasmic reticulum of bitopic integral membrane proteins as well as CPP²⁶ provide strong support for this notion. Furthermore, the efficiency of integration of CPP into the heterologous microsomal vesicles, as assayed by the resistance to alkaline extraction, can be increased to almost 100% by the addition of these purified proteins to the *in vitro* translocation system²⁶.

A portion of this work has been presented elsewhere²⁷. We thank Drs J. Hemperly and B. Cunningham for use of, and assistance with, their Beckman sequencer. This work was supported by grants from the NIH (GM 27155 and AM 14632) and the Muscular Dystrophy Association of America. P.D. is an investigator of the American Heart Association, Tennessee affiliate.

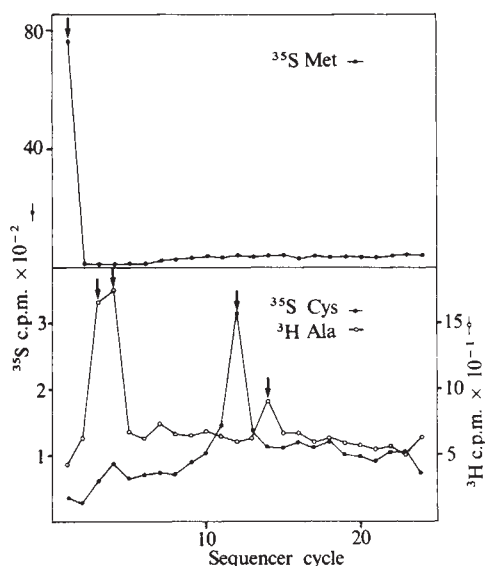


Fig. 2 Partial amino-terminal sequence determination of CPP translated *in vitro* in the absence of microsomal membranes. Translations (1 ml) were performed using either ³⁵S-methionine (1,000 Ci mmol⁻¹) or ³H-alanine (75 Ci mmol⁻¹) and ³⁵S-cysteine (700 Ci mmol⁻¹) (all NEN). Citrate synthase and oxaloacetate were present in the translation mixture to prevent the acetylation of the amino terminus of CPP³⁰. CPP was immunoprecipitated, separated by gel electrophoresis, eluted from the gel and sequenced¹⁵.

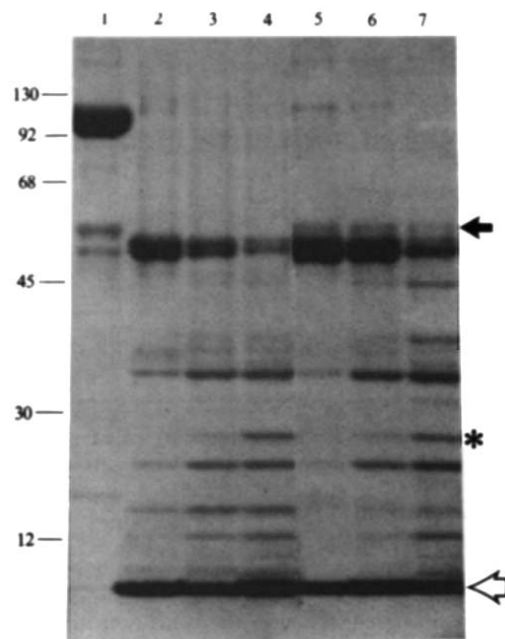


Fig. 3 Tryptic digestion of CPP in the absence or presence of Triton X-100. All samples were analysed by electrophoresis as described in Fig. 1 legend and the gel was stained with Coomassie blue. Each digestion contained, in a volume of 50 μl, 50 μg (protein) of purified SR vesicles, 1 M sucrose, 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5, and the indicated amount of trypsin, pretreated with TPCK. Lanes 2-4 also contained 1% Triton X-100. Trypsin concentrations were: lane 1, 0 μg ml⁻¹; lanes 2 and 5, 0.6 μg ml⁻¹; lanes 3 and 6, 6 μg ml⁻¹; and lanes 4 and 7, 60 μg ml⁻¹. Digestions were carried out for 45 min at 23 °C and terminated by the addition of 10 μl Trasylol. Solubilization of the SR vesicles by Triton X-100 was ascertained by the inability of the detergent-solubilized vesicles to be pelleted by centrifugation conditions which were calculated to pellet particles with a sedimentation rate >40 S; intact SR vesicles were quantitatively sedimented by these conditions (data not shown). The band indicated by the solid arrow is caseinogen, a peripheral membrane protein located in the lumen of the SR. It was protected from digestion by the intact vesicles (lanes 5-7) but not when the vesicles were solubilized by detergent (lanes 2-4). The asterisk indicates the position of trypsin. The heavy band indicated by the open arrow is Trasylol. The positions of the molecular weight markers are indicated as in Fig. 1.

Received 2 September 1980; accepted 11 March 1981.

- MacLennan, D. H. & Holland, P. C. *Rev. Biophys. Bioengng.* **4**, 377-404 (1975).
- Fleischer, S., Wang, C.-T., Saito, A., Pilarska, M. & McIntyre, J. O. in *Cation Flux Across Membranes* (eds Mukohata, Y. & Packer, L.) 193-205 (Academic, New York, 1979).
- Rizzolo, L. J., le Maire, M., Reynolds, J. & Tanford, C. *Biochemistry* **15**, 3433-3436 (1976).
- Thorley-Lawson, D. A. & Green, N. M. *Eur. J. Biochem.* **40**, 403-413 (1973).
- Rizzolo, L. J. & Tanford, C. *Biochemistry* **17**, 4044-4048 (1978).
- Saito, A., Wang, C.-T. & Fleischer, S. *J. Cell Biol.* **79**, 601-616 (1978).
- Klip, A., Reithmeier, R. A. F. & MacLennan, D. H. *J. Biol. Chem.* **255**, 6565-6568 (1980).
- Allen, G., Trinaman, B. J. & Green, N. M. *Biochem. J.* **187**, 591-616 (1980).
- Lingappa, V. R., Katz, F. N., Lodish, H. F. & Blobel, G. *J. Biol. Chem.* **253**, 8667-8670 (1978).
- Ploegh, H. L., Cannon, L. E. & Strominger, J. L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2273-2277 (1979).
- Bonatti, S. & Blobel, G. *J. Biol. Chem.* **254**, 12261-12264 (1979).
- Blobel, G. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1496-1500 (1980).
- Reithmeier, R. A. F., de Leon, S. & MacLennan, D. H. *J. Biol. Chem.* **255**, 11839-11846 (1980).
- Allen, G. *Biochem. J.* **187**, 545-563 (1980).
- Lingappa, V. R., Devillers-Thiery, A. & Blobel, G. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2432-2436 (1977).
- Greenway, D. C. & MacLennan, D. *Can. J. Biochem.* **56**, 452-456 (1978).
- Chyn, T. L., Martonosi, A. N., Morimoto, T. & Sabatini, D. D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1241-1245 (1979).
- Vanderkooi, J. M., Ierokomas, A., Nakamura, H. & Martonosi, A. *Biochemistry* **16**, 1262-1266 (1977).
- Wang, C.-T., Saito, A. & Fleischer, A. *J. Biol. Chem.* **254**, 9209-9219 (1979).
- le Maire, M., Möller, J. V. & Tanford, C. *Biochemistry* **15**, 2336-2342 (1976).
- Steck, T. L. & Yu, J. *J. supramolec. Struct.* **1**, 220-232 (1973).
- Shanahan, M. F. & Czech, M. P. *J. Biol. Chem.* **252**, 6554-6561 (1977).
- Neubig, R. R., Krodol, E. K., Boyd, N. D. & Cohen, J. B. *Proc. natn. Acad. Sci. U.S.A.* **76**, 690-694 (1979).
- Walter, P., Jackson, R. C., Marcus, M., Lingappa, V. R. & Blobel, G. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1795-1799 (1979).
- Walter, P. & Blobel, G. *Proc. natn. Acad. Sci. U.S.A.* **77**, 7112-7116 (1980).
- Anderson, D. J., Mostov, K. E., Walter, P. & Blobel, G. (in preparation).
- Mostov, K. E., DeFoor, P., Fleischer, S. & Blobel, G. *Europ. J. Cell Biol.* **22**, 147 (1980).
- DeFoor, P. H., Levitsky, D., Biryukova, T. & Fleischer, S. *Archs Biochem. Biophys.* **200**, 196-205 (1980).
- Shields, D. & Blobel, G. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2059-2063 (1977).
- Schmidt, G. W., Devillers-Thiery, A., Desruisseaux, H., Blobel, G. & Chua, N.-H. *J. Cell Biol.* **83**, 615-622 (1979).

MATTERS ARISING

Flexural strength of cements

BIRCHALL *ET AL.*¹ are to be congratulated on the improvements made in the flexural strength of cement, however, we question their scientific explanation which is given in terms of the removal of large size pores $\geq 100 \mu\text{m}$; it is postulated that these pores are simple strength controlling Griffith flaws. We have shown² that although notched specimens of ordinary cement paste tested in flexure seem to behave according to simple Griffith theory for specimens having a fixed beam depth, nevertheless, when the beam depth is altered different values are obtained for the fracture energy, R . In fact the apparent value of R increases with increasing specimen depth and R is not therefore behaving as a material constant, and a simple application of the Griffith equation is not acceptable. It is apparent from their conclusions that Birchall *et al.* have carried out measurements on beams of fixed overall depth. Note that in our experiments, specimens of differing size were cut from the same cured samples of cement and therefore the variation in R values could not result from changes in specimen microstructure from one sample to another. By extrapolation we deduced the behaviour of apparently sharp flaws in large cement samples and showed that the material can be regarded as intrinsically weak, for example, for a w/c ratio of 0.3 having an intrinsic strength of $\sim 15 \text{ MPa}$, and that sharp flaws have an effective radius of curvature of $\sim 0.5 \text{ cm}$; therefore flaws $\leq 0.5 \text{ cm}$ in size will have a comparatively small effect on strength.

To improve strength it is necessary to increase the intrinsic strength by increasing the total number of effective chemical bonds per unit area of the material for example by reducing the total amount of unfilled space in the material, that is increase density. Birchall *et al.* quote a value for elastic modulus of 40 GPa for improved (MDF) cement—about twice that of OPC which suggests that its basic structural properties have been changed. We conclude therefore that it is a little misleading to attribute the strength increase solely to the elimination of large flaws and that the high strength has been achieved by a reduction in w/c ratio, good packing, compaction and drying out, all of which are expected to increase strength. The resulting MDF cement, although intrinsically stronger, is sensitive to flaws and will need to be free of macroscopic flaws to achieve the higher strengths; for example a 1-mm flaw will reduce the strength of MDF cement by a factor of 3, whereas a similar size flaw in ordinary Portland cement $w/c = 0.3$ will only reduce the strength by a factor of 1.6. Therefore, flaws must be eliminated dur-

ing processing. However, as discussed above, simply eliminating flaws from an ordinary cement paste would not result in strengths as high as those quoted for the MDF cement.

J. E. BAILEY

Department of Metallurgy and
Materials Technology,
University of Surrey,
Guildford, Surrey GU2 5XH, UK

D. D. HIGGINS

Cement and Concrete Association,
Wexham Springs,
Slough SL3 6PL, UK

1. Birchall, J. D., Howard, A. J. & Kendall, K. *Nature* **289**, 388–390 (1981).

2. Higgins, D. D. & Bailey, J. E. *J. mater. Sci.* **11**, 1995–2003 (1976).

BIRCHALL *ET AL.*¹ have shown that the flexural strength of a Portland cement paste can be increased to $\sim 65 \text{ MPa}$. They termed the modified paste as “macro-defect-free” (MDF) paste and attributed the improvement to the removal of macro-defects. However, this interpretation may be queried.

Birchall *et al.* have observed that the flexural strength data of both the MDF paste and the ordinary Portland cement paste could be fitted to the Griffith equation.

$$\sigma = (ER/\pi C)^{1/2}$$

where “tensile cracking stress (σ) is related to an effective critical crack length (C) which may be a pore; Young’s modulus (E) and fracture surface energy (R) being taken as material constants”. They found that E and R are 20 GPa and 19 J m^{-2} for an ordinary Portland cement paste and 40 GPa and 30 J m^{-2} for the MDF paste even though both the pastes have very similar porosities.

It appears that both the material constants E and R are so dependent on the pore size distributions that their product could be altered by a factor of 3. In that case one may wonder how the Griffith equation could be applied to these pastes. This uncertainty has been further emphasised in their Fig. 3 which shows that the MDF paste has higher strengths than the ordinary Portland cement paste even when both pastes have similar sized critical flaws. More probably Birchall *et al.* have altered the nature of the paste altogether by using rheological aids such as plasticizers and superplasticizers, which are generally organic products of some chain lengths; some even contain organic polymers. The addition of polymers to a cement paste increases its flexural strength as well as its flexural/compressive strength ratio from ~ 0.1 for a brittle failure to a higher value characteristic of a plastic failure. Note that the MDF paste

has also a flexural/compressive strength ratio of 0.3 (65/200).

It will not be very difficult to ascertain experimentally if the nature of the paste has been modified in which case the observed improvement may have little to do with the removal of macro-defects.

Note added in proof: The patent application by Birchall *et al.*² shows that polymers have been used by them.

S. CHATTERJI

Byggeteknik,
Teknologisk Institut, Gregersensvej,
DK-2630, Tastrup, Denmark

1. Birchall, J. D., Howard, A. J. & Kendall, K. *Nature* **289**, 388–390 (1981).

2. Birchall, J. D., Howard, A. J. & Kendall, K. *European Patent Application No.* 80301909.

BIRCHALL *ET AL.* REPLY—Bailey, Higgins and Chatterji have raised interesting questions, some perhaps prompted by the lack of detail in our short paper¹. We are aware of the detailed arguments relating to the fracture behaviour of cement paste and of the careful work by Bailey and Higgins on this. Our proposition is that, bearing in mind the heterogeneous and inconstant nature of cement paste, the Griffith criterion is an adequate explanation of the failure behaviour of the material. It has the advantage of predicting what steps should be taken to improve the material—the removal of macroscopic flaws—and our paper shows that this is demonstrably effective.

As Bailey and Higgins correctly point out, fracture tests on ordinary cement paste give a spread of values of R , the fracture energy, a parameter which appears to change significantly with test geometry and crack speed among other variables. We believe that these variations in R make it impossible by conventional experiments to distinguish the Griffith theory from more complex theories of fracture. The experimental scatter is too great. Essentially, what we have done is to extend the study into an unexplored range of flaw size, down to $10 \mu\text{m}$, when the overall behaviour is seen to fit the Griffith theory satisfactorily.

It is suggested that the high value of elastic modulus (40 GPa) quoted in our paper for MDF cement indicates a higher number of bonds per unit area or a higher bond strength and a microstructure fundamentally different from that of normal paste. It is indeed implicit in our understanding of the mechanism of hydration and setting², that hydrate morphology will vary with the volume of space to be filled and hence with packing, and so on. However, a major point in our paper is that whereas elastic modulus is dominantly related to total porosity volume, the flexural strength is

dominantly related to the size and proportion of macroscopic voids and, as illustrated in Fig. 4, is insensitive to total porosity volume. Thus high flexural strength is observed in specimens having a range of values of elastic modulus, 25–40 GPa.

Chatterji suggests that the properties of MDF cement are those of a polymer-modified paste. However, the strength values of MDF cement far exceed those claimed for pastes made at modest temperature and pressure in the presence of a water-reducing additive, with polymer addition or by polymer impregnation. Furthermore, the failure mode of MDF cement is brittle with no evidence of plastic deformation. Thus the improved properties of MDF cement are attributable to the altered pore size distribution of the inorganic matrix and are, as we indicate, independent of the chemical nature of the inorganic cement matrix. In this context, other work (in preparation) has shown that MDF cements can indeed be further modified to increase significantly the fracture toughness, with the result that flexural strength is raised still further to levels exceeding 150 MPa. The absence of macroscopic voids is an essential prerequisite for the attainment of such very high strength.

J. D. BIRCHALL
A. J. HOWARD
K. KENDALL

Technical Department,
ICI Mond Division,
Runcorn, Cheshire WA7 4QD, UK

1. Birchall, J. D., Howard, A. J. & Kendall, K. *Nature* **289**, 388–390 (1981).

2. Birchall, J. D., Howard, A. J. & Bailey, J. E. *Proc. R. Soc. A* **360**, 445–453 (1978).

Can passive tactile perception be better than active?

PASSIVE tactile perception has invariably been found to be equal to or inferior to active tactile perception^{1–3}. An exception has recently been reported in an experiment by Magee and Kennedy⁴ who guided the finger of each blindfolded subject around raised-line drawings of common objects. These (passive) subjects were able to identify more of the drawings than the active subjects whose fingertip movements were unguided. Magee and Kennedy suggest that “the act of planning may draw on the limited resources of the haptic system so that less processing capacity is available to monitor and distinguish relevant from irrelevant kinaesthetic input”.

We consider this to be an implausible explanation of active inferiority for the following reasons:

(1) The “act of planning” is more cognitive than sensory. No evidence is provided

to support the idea that, in this experiment, such planning can be expected to interfere with (or draw on limited resources of) the haptic (sensory) system.

(2) The resources of the haptic system must be limited at some level of task complexity but the job it was required to do in this experiment should be well within its capacity. When all fingers are simultaneously engaged in palpating an object, the channel is apparently not overloaded. Why should movements on one fingertip tax the system to the degree that planning of such movements proves too much? Moreover, Shiffrin *et al.*⁵ present rather convincing evidence for an “unlimited-capacity nonattentional model of tactile perceptual processing”.

(3) Presumably, “relevant” kinaesthetic input comes from movements made when the fingertip faithfully follows the raised line and “irrelevant” inputs from errors (straying from the line). But surely deviations are important error signals which guide ensuing movements rather than act as “irrelevancies”. Information is gained either way.

(4) The comparison being made was not simply between active and passive haptic conditions. Rather, because haptic information was so (unusually) impoverished, conscious attention was necessary to build up a “picture”—for both passive and active subjects—but active subjects suffered the disadvantage of also having to plan movements.

In a different experiment, it was shown that when passive and active subjects are yoked in a tactile maze exploration task, the performance of active subjects suffers because they have the responsibility of regulation (planning) of movements⁶. This experiment, and that of Magee and Kennedy, showed that it is possible to demonstrate interference at the cognitive level when information is transmitted by the haptic system—not that passive tactile performance is better than active.

BARRY L. RICHARDSON
DIANNE B. WUILLEMIN

Department of Psychology,
University of Papua New Guinea,
Port Moresby, Papua New Guinea

Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

1. Gibson, J. J. *Psychol. Rev.* **69**, 477–491 (1962).
2. Landrigan, D. T. & Forsyth, G. A. *J. exp. Psychol.* **103**, 1124–1130 (1974).
3. Schwartz, A. S., Perey, A. J. & Azulay, A. *Bull. Psychonomic Soc.* **6**, 7–9 (1975).
4. Magee, L. E. & Kennedy, J. M. *Nature* **283**, 287 (1980).
5. Shiffrin, M. R., Craig, C. J. & Cohen, E. *Percept. Psychophys.* **13**, 328–336 (1973).
6. Richardson, B. L., Willemin, D. B. & MacKintosh, G. T. *Br. J. Psychol.* (in the press).

MAGEE AND KENNEDY REPLY—Richardson and Willemin argue that our experiments did not establish superior passive tactile perception in the identification of raised-line drawings. They base their claim on studies with tactile finger mazes in which they found passive exploration to be superior to active exploration, and were then able to show that passive superiority was due to a cognitive factor, that is, the act of planning exploratory activity. They argue that passive superiority with raised-line drawings is also due to this cognitive factor.

Modern theorists recognize that planning and anticipation are crucial to perception, and we too recognize this as a critical component of haptic perception. However, the crucial problem for present purposes is that Richardson and Willemin's conclusion is based on superficial similarity between two types of tasks rather than on fundamental principles of haptic perception.

Clearly, Richardson and Willemin's finger-maze learning could be solved as a verbal sequence of eight discrete left-right turns¹, a number within the bounds of short-term memory. In contrast, the identification of raised-line drawings involves complex shape information that is not discrete. Our subjects had to deal with a line continuously varying in location, direction and extent, that is, the task required the subject to integrate across time subtle spatial information that was difficult to encode verbally. Integrating all this information was not easy, as shown by the generally low levels of performance on this task. These low levels of performance indicate (contrary to Richardson and Willemin's assertion, made without supporting evidence) that our single-finger task was not facile and was not well within haptic capacities. Richardson and Willemin's task has little to do with perception, while identifying shape and form is the *sine qua non* for haptic perception.

Our task was one of haptic perception in which we have shown passive superiority.

LOCHLAN E. MAGEE
JOHN M. KENNEDY

Defence and Civil Institute
of Environmental Medicine,
PO Box 2000, Downsview,
Ontario, Canada M3M 3B9

1. Richardson, B. L., Willemin, D. B. & MacKintosh, G. T. *Br. J. Psychol.* (in the press).

BOOK REVIEWS

The genius phenomenon

Donald Michie

John von Neumann and Norbert Wiener: From Mathematics to the Technologies of Life and Death. By Steve J. Heims. Pp.547. ISBN 0-262-08105-9. (MIT Press: 1980.) \$19.95, £14

THE appearance of S. J. Heims's study of John von Neumann and Norbert Wiener is opportune. Technologies of computing, communication and control sprang from where the two men sowed, and are now taking us beyond range of our charts. Will the cybernetic age resemble other ages? Will scientists still be "on tap but not on top"? Or did the post-war influence exerted by physicists and mathematicians in American military circles presage new responsibilities for a stratum of society commonly regarded as temperamentally ill-equipped to bear them?

If so, it seems that we may have to accustom ourselves to receiving guidance from our emotional juniors on matters of life and death. The saying that "genius is to madness near allied" was always of course misleading. But if one were to decide to call infants mad for their helpless and grasping self-absorption, and adolescents mad because of their lonely assertiveness and intensity, then we could read meaning into the saying after all. The association between one or another form of emotional underdevelopment and extreme manifestations of creative excellence has often been remarked. It finds fascinating exemplification in Dr Heims's book about information science's two great founders.

Norbert Wiener no doubt started life as an infant; but if by some mischance he did not, then he later repaired the omission. Thus, Bertrand Russell, writing in 1913:

At the end of September an infant prodigy named Wiener, Ph.D. (Harvard), aged 18, turned up with his father who teaches Slavonic languages there, having first come to America to found a vegetarian communist colony, and having abandoned that intention for farming, and farming for the teaching of various subjects, [say] mathematics, Roman Law, and mineralogy, in various universities. The youth has been flattered, and thinks himself God Almighty — there is a perpetual conflict between him and me as to which is to do the teaching.

Socially unsure to the end of his life, Wiener's child-like attributes of generosity and utopian optimism struck all who knew him. There were also irritants — absent-mindedness, simulation of sleep during the lectures or conversations of others, and the constant and overwhelming hunger for praise. The latter would impenitently break surface even when to all appearances it had

been totally transcended. A friend recalled a meal towards the end of Wiener's life:

... Wiener stopped and turned the conversation to Yilmaz (a physicist). Now people say that Wiener wasn't good at listening to people. Wiener was perfectly quiet as Yilmaz expounded his color theory ... then we went on to invariance, and science and epistemology; the conversation then took on a very general tone. I remember it was a beautiful, sunny day; it was a very heady experience; we were really getting along perfectly. Then at the end Wiener said to me: 'Was I a good listener?'

I met Norbert Wiener at Oxford around 1950. N. A. Mitchison and I, with other graduate students, took him to Mitchison's rooms in Magdalen after he had talked to an undergraduate society. We settled him in an arm-chair, where he seemed to fall at once into a deep sleep. We began to discuss genetics among ourselves. This was a year or two before Crick and Watson had identified the genetic material in people's minds with DNA and had proposed the existence of a coding problem. Wiener's eyes opened briefly: "How many bits of information, I wonder, are coded in the human chromosomes? Could one, do you think, get an upper bound by chemical arguments?". He closed his eyes. We exchanged glances, and having missed the whole of a very extraordinary point resumed our aimless talk.

Wiener's youthful attention was drawn by Russell to Einstein's 1905 paper on Brownian motion. Marc Kac wrote after Wiener's death:

In retrospect one can have nothing but admiration for the vision which Wiener had shown when, almost half a century ago, he had chosen Brownian motion as a subject of study from the point of view of integration. To have foreseen at that time that an impressive edifice could be erected in such an esoteric corner of mathematics was a feat of intuition not easily equalled now or ever. ... It gave us not only a new way of looking at problems but actually a new way of thinking about them.

The man who in the late 1940s founded cybernetics, supervised in the early 1960s a PhD student in a further development of this early work on stochastic processes. The student, Michael Arbib, has gone on to establish at the University of Massachusetts a Centre for Systems Neurosciences — coherent embodiment of Wiener's dream of a mathematical school for the analysis of biological organization and its control. Every great scholar hopes to leave more than his writing. Wiener, in surveying today's work on formal models of brain function, would I think be satisfied both with the project itself and with its humanistic goals.



Norbert Wiener (top) and John von Neumann. Both "loved to dazzle and [were] preternaturally equipped to do so".

From first to last Wiener followed Descartes: "I would not engage in projects which can be useful to some only by being harmful to others". His penalty was to be the eternal maverick — "calf or other animal not marked with an owner's brand".

Von Neumann's consuming drives presented a contrast to all this. Like Wiener, he loved to dazzle and was preternaturally equipped to do so: Leo Wigner referred to his brain as "a true miracle". But he made sure to dazzle as an insider. In the higher counsels of the Pentagon he showed a demonstrative hawkishness beyond that of the less rootless, less

intellectual, professional hawks of the military. In 1950 he was remarking: "If you say why not bomb them tomorrow, I will say why not today? If you say today at 5 o'clock, I say why not one o'clock?"

In connection with von Neumann's advocacy of preventive nuclear war, Steve Heims writes of his response when asked by Ulam to explain the "Hungarian phenomenon":

... he would say that it was a coincidence of some cultural factors which he could not make precise: an external pressure on the whole society of this part of Central Europe, a sub-conscious feeling of extreme insecurity in individuals, and the necessity of producing the unusual or facing extinction.

Edward Teller remarked of his youth that Hungary was foundering and if he wanted to survive he would "have to be better, much better than anyone else", echoed by another scientifically spectacular émigré, Davis Gabor: "Innovate or die!"

The Hungarian phenomenon may or may not be overemphasized in this book. But as concerns John von Neumann's desire to be not just on tap, but on top, Heims is replete with vivid documentation. A picture takes form of breath-taking intellectual exuberance and power lodged in a coldly ambitious yet sanguine personality, both complex and strangely undeveloped. When the author writes, in connection with the creation of nuclear weapons, of von Neumann's "compelling desire to serve and be part of the élite establishment, and to assume a modern American role reminiscent of the court astrologer or court engineer of a feudal military empire", we recognize it for the truth. We also recognize that von Neumann himself in all likelihood would jauntily have accepted the label for ornament. Among Johnny's attributed sayings was: "Only a man from Budapest can enter a revolving door behind you and emerge ahead of you".

Wiener's tangible legacy groups itself around the foci of neurobiology and cognitive science. Von Neumann's is to be found in the technology of brute-force computing, rather than in today's developments in artificial intelligence. Although aware of A. M. Turing's contributions to logic, to computer design and to the mechanization of human thought processes, von Neumann showed little sign of having received an influence. The notion of resource-limited computation, key to the brain's cognitive processes and their machine simulation, would surely have been alien to one whose reluctance to admit limits to his own calculational powers became a byword. A similar assumption of omniscient rationality lies at the root of the game-theoretic model of economic behaviour which was erected by him and Morgenstern, now displaced by H. A. Simon's notion of practically feasible, as opposed to ideal, calculations of self-interest.

In one respect, Heims's book follows a popular error. Von Neumann is credited with sole intellectual rather than political authorship of the EDVAC design. This doubtless arises from the circumstance that the report of the EDVAC committee, which included Eckert and Mauchly, bore the sole signature of its chairman.

In general, however, Steven Heims's scholarship is both voluminous and detailed, and his personal biases, which are those of a liberal humanist, are not too obtrusive. The author succeeds least in his attempted tour of the pure and applied mathematics which these men built, in von Neumann's case outstanding in this

century. For this we must wait for another book. But the living substance of two great minds and of the convulsive epoch through which they lived is here recreated in 414 pages of narrative and 115 of notes. The book informs, stimulates and entertains. In its cumulative effect it also profoundly disturbs. It is right that we scientists should be induced to contemplate our professional responsibility for the repercussions of our trade. The labours of Dr Heims have fashioned a powerful instrument to this end. □

Donald Michie is Professor of Machine Intelligence in the University of Edinburgh.

New dimension for archaeology in tool use

Ruth Tringham

Experimental Determination of Stone Tool Uses: A Microwear Analysis. By L. Keeley. Pp.320. ISBN hbk 0-226-42888-5; ISBN pbk 0-226-42889-3. (University of Chicago Press: 1980.) Hbk \$15, £10.50; pbk \$7, £4.90.

AMONG the spate of literature accompanying the rise of interest in prehistoric artifact use, two books stand out: *Lithic Use-Wear Analysis*, edited by Brian Hayden (Academic, 1979), and this volume by Lawrence Keeley.

Experimentation forms the most important aspect of Keeley's research, and it comprises the third and fourth — by far the most important — chapters of his book. The two preceding chapters discuss recent research on microwear and the method of the microscopic examination of the edges of stone tools.

For Keeley, experimentation is the "fabrication of replicas of archaeological implements and their use on various materials under controlled conditions" (p.3), the ultimate purpose of which is "the interpretation of particular archaeological pieces usually from archaeological occurrences limited in time and space" (p.5). The value of such replications is that they help to bring alive long-vanished patterns of behaviour, and suggest directions for more systematic research. The series of replications reported by Keeley in detail describe the formation of polishes on the surfaces and edges of flint flakes as a result of contact with different materials in different tasks. These polishes are then identified on archaeological specimens and inferences made about the material and the task of the prehistoric tool.

Keeley's replications, however, have limited implications for general issues of the evolution of human behaviour. In fact they are not really "experiments" in the definition of the term used in experimental sciences, in that they are not designed to test empirical hypotheses by the systematic and artificial manipulation of variables.

They do not explain the nature of the properties of the raw materials being tested. As Keeley is the first to point out (p.167), his replications do little to explain the variation in the character of the polishes formed on the flint. On the other hand, he criticizes as artificial such experiments as my own which do not replicate purposeful tasks but, under laboratory conditions, mechanically bring tool edge and worked material into contact in a repeated and standardized action. And yet to criticize such experiments as "artificial" misses the whole point of experimentation.

In spite of this inherent theoretical weakness in the book, there is no doubt that Keeley has made a methodological breakthrough in microwear studies by focusing on an essential aspect of the reconstruction of the utilization of stone tools — the correlation between the variability of polishing of flakes and that of the worked material.

The second major section (Chapters 5–7) reports the observations which Keeley made on prehistoric tools and the application of this microwear information to the interpretation of variability in three Lower Palaeolithic assemblages from southern England (Clacton, Swanscombe and Hoxne.) Here, as stated earlier in the book, Keeley's main aim is to reconstruct task-specific behaviour in his sites and assemblages. Yet his conclusions from microwear observations of the three assemblages do not directly address this problem. The observations provide information on the utilization of rock as a raw material resource rather than on the activities which the tools performed. On the basis of microwear information, Keeley concludes that the utilization of flint as a source of tool raw material was opportunistic and relatively unplanned in the Clactonian and Acheulian industries represented by his three sites. Selection of flakes for specific tasks, if such selection existed, was on the basis of the shape and

angle of the edge, not on the shape and size of the flake itself. There is a most interesting discussion on the relationship between the role of hand-axes and flake tools in the stone tool assemblages, which of course is pertinent to our understanding of the changing use of stone as a raw material between the Lower and Middle Palaeolithic industries, hand-axes being much rarer in the latter. It is unfortunate that so far very little microwear information has been collected on hand-axes. As Keeley points out, "there is much potential for future research".

The implication of Keeley's conclusions in this section is that microwear data can provide unique information on the use of stone by allowing us to relate empirically the form of artifacts to their utilized parts. Furthermore, such data can provide the sole source of information on an aspect of the evolution of human behaviour which has received little discussion by archaeologists — the consumption of resources: their uses, maintenance and discard. But it seems that Keeley does not recognize this broader significance of his work.

Similarly, the two concluding chapters are somewhat disappointing. They discuss, respectively, areas for future research and the conclusions of the study so far, and both are characterized by a surprising modesty and lack of ambition. The conclusions of the microwear analysis of the prehistoric assemblages which Keeley (p.176) feels are the most important are that it has "... uncovered many fascinating details about the technology of the Lower Palaeolithic, not the least of which are the existence of plant 'gathering' knives and information about the methods of hide preparation employed"; furthermore, it has "uncovered some interesting relationships between the morphology and function of implements in the assemblages . . .", and it has provided a "little tantalizing information pertinent to questions concerning the purposes of hand-axes in the Middle Acheulian . . .". But is microwear analysis — all those hours and energy — destined to provide only the "fascinating", the "tantalizing" and the "interesting"? Likewise, the suggestions for future research tend to concentrate on particular details and problems.

In this book there is an enormous amount of valuable experimental and analytical information, which should have an impact on a wide public. Keeley warns about many of the traps of microwear analysis, including over-confidence and over-optimism. He fails, though, to mention the most dangerous one of all: that one can fall in love with one's flake edges, especially when they are viewed under the microscope, and forget why it is that we originally decided to spend so much time with them. □

Ruth Tringham is Associate Professor of Anthropology at the University of California at Berkeley.

Natural history from Henry VII to Victoria

G. D. R. Bridson

British Natural History Books, 1495-1900: A Handlist. By R.B. Freeman. Pp.437. ISBN 0-7129-0971-0/0-208-01790-0. (Dawson, Folkestone, Kent/Archon, Hamden, Connecticut: 1980.) £20, \$39.50.

MODEST proportions and unpretentious presentation combine to disguise this book's place as a milestone in the progress of British natural history bibliography. We have never had an equivalent of, for instance, Meisel's magnificent bibliography of American natural history down to 1865, though such works as Simpson and Henrey for botany, and Irwin and Lisney for ornithology and entomology, have made significant contributions. Otherwise one could turn to the German compilations of Engelmann, Carus and Taschenberg for a bibliography of general natural history and zoology down to 1880, amongst which is buried British material.

The compilation of a checklist of all British natural history books is a task for the experienced bibliographer and we are fortunate that one so well qualified as Dr Freeman should attempt the task. The result is a work that does great credit to him and outstanding service to the user. It is only a handlist but within that format lies much concealed scholarship and great attention to accuracy. This is enhanced by providing the full forenames of every author possible, by searching out and recording multiple editions, by providing a chronology of pre-1801 titles and by indicating in the index "those works which, in the opinion of the author, are the most important contributions to the subject of the entry". The 35-page subject index extends the value of the book beyond that of a handlist, though that still remains its basic function and as such it is remarkably comprehensive. One can find omissions among its 4,206 entries by cross-checking with works such as Jackson's guide to botanical literature or Taschenberg's general natural history listings. But, I must stress, so much is included that only specialists in book rarities will find fault.

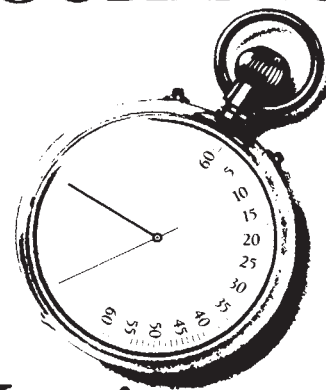
What is more controversial is the exclusion of papers in periodicals and, unfortunately, any bibliographical

reference to the periodicals themselves. This a handlist of books, but just when a preprint, offprint, reprint or plain tear-out becomes a separate book will give rise to much argument in many individual cases. So many papers from periodicals were reprinted, often with new pagination, in the nineteenth century that the full record of this transition literature will take many years to document with accuracy. The exclusion of bibliographical mention of periodicals is, in my view, the book's most serious omission and one that would not have required so much effort to rectify.

Librarians, bibliographers, historians, book-collectors, booksellers and naturalists will all want this work on their shelves for frequent reference, and several of those will wish to interleave and annotate their copies. They will find few typographical errors to correct, an odd binary entry to delete perhaps (for example Nos. 439 and 1,181), and a few cross-references to add here and there. At £20 the book is rather costly for such commonplace presentation but in terms of intrinsic value and likely duration of utility that cost will be amply rewarded. □

G. D. R. Bridson is Librarian at the Linnean Society of London.

IRCS JOURNALS



Keeping pace with science

IRCS Medical Science
St. Leonard's House
St. Leonardgate,
Lancaster, U.K.

A member of Elsevier Science Publishers

In celebration of the centenary of the British Museum (Natural History), the Museum and Cambridge University Press have published *Chance, Change and Challenge*, a two-volume illustrated compilation which will be of particular interest to sixth-formers and undergraduates. Volume 1, *The Evolving Earth*, deals with the origin and development of the Earth, and Vol. 2, *The Evolving Biosphere*, examines the evolution of life. Prices are: Vol.1 hbk £30, pbk £10.50; Vol.2 hbk £32.50, pbk £11.50.

Howard Hinton's eggs — a monumental bequest for entomology

Charles Neville

Biology of Insect Eggs. By H. E. Hinton. Pp.1,125. Three volumes. ISBN 0-08-021539-4. (Pergamon: 1981.) \$400, £167.

THIS posthumous work is monumental in both senses of the word. Professor Hinton tried courageously to finish it during his terminal illness, but the final stages of publication, including compilation and checking of the extensive references and index, had to be completed by his research assistant, Mrs Joyce Ablett, and by Dr Colin Mapes. Information on insect eggs was previously scattered throughout the literature. It now becomes available in one source, which is certain to become the standard work on the subject. But these three volumes constitute no mere review; they also contain an enormous amount of original information, based upon a lifetime of work divided between field and laboratory.

The egg stage of insects is no less important than those which follow. Yet most entomologists would be content if they could recognize even the adult stage of those species of flies found on cow pats. Howard Hinton could, however, with the aid of a hand lens, identify their eggs down to species *in situ*. As might be expected from one of the world's most knowledgeable entomologists, the text is packed with interesting examples of evolutionary adaptations.

Some of the most fascinating arise from the fact that, being small, insect eggs have a relatively large surface area. Whereas this is an advantage for oxygen uptake, it means that terrestrial eggs are prone to desiccation; conversely, eggs in aquatic surroundings are threatened by drowning. Professor Hinton shows how these factors explain the greater complexity of insect eggshells compared with those of larger animals, and relations between respiratory structure and function receive extensive coverage in the first volume. The inner layer of insect eggshells is filled with air, and it communicates with the outer surface through channels (aeropyles), some of which open on elevated turrets, or even horns. In some eggs, which might otherwise drown, trapped air bubbles function as gills which both provide oxygen themselves and give access to a further supply from the surrounding water. Such bubbles eventually shrink, however, and need replenishment. This is fine for motile stages but not for eggs, which cannot move. To solve this problem, some eggs which are laid in dung begin respiration by a shrinking bubble, and then change over to a constant-volume bubble trapped between hydrofuge hairs (a respiratory plastron). Hinton has shown how there are even more examples of plastrons in terrestrial eggs than amongst aquatic larvae. Plastrons are particularly

appropriate for eggs which are subjected to alternate drying and flooding, and have independently evolved in unrelated species many times. A further chapter describes how some eggs can absorb water via special organs (hydropyles).

Subsequent chapters in the first volume cover the interrelations between adults and their eggs (fecundity, oviposition and parental care). There are also chapters on the adult glands which secrete protective egg cases; on the proteins which form them; on eggshell proteins and their chemical cross-linking; and on how eggs interact with their enemies, often fooling them with deceptive colours or shapes.

The second volume describes the egg structure and adaptations of insects, order by order. Numerous keys to identification will prove invaluable to future workers,

though, sadly, there was not time for a chapter on fleas. These two volumes are profusely illustrated: I counted 911 photographs, mostly original scanning electron micrographs, a technique which Howard Hinton used right from its introduction. In addition there are 32 tables and nearly 300 clear text diagrams, most of which are original.

The final volume provides the overwhelming bibliography (over 5,000 references) and separate indexes to species, authors and subjects. The work ends with a list of Professor Hinton's publications: these three volumes now complete it. His contributions to entomology thus end on a resounding note. □

Charles Neville is Reader in Zoology at the University of Bristol.

Children who are left to themselves

P. E. Bryant

Developmental Psychology and Society. Edited by John Sants. Pp.389. ISBN 0-333-21340-8/0-312-19751-9. (Macmillan Press, London/St Martin's Press, New York: 1980.) £20, \$35.

D. W. WINNICOTT, a well-known child psychiatrist, is quoted as having remarked in a heated discussion that there is no such thing as a baby. He meant that it is absurd to treat babies in isolation. They are in constant contact with parents and others, and most of the significant things that they do are social in some way or other. This is a point which most child psychologists accept nowadays, but they still find it hard to incorporate into their work.

The clearest example is the question of the importance of language in intellectual development. It seems obvious that people talk to children and tell them things, and that this contributes significantly to the child's growing understanding of his world. Yet the effects of this sort of communication have been virtually ignored by child psychologists. Some, like Piaget, go to great lengths to show that language is of small importance to a child's development; others think that it might have some effect but in the form of "inner speech" — the child talking to himself. Nobody seems to realize that grown-ups actually talk to children. As usual, the social element is left out.

The great merit of the book which John Sants has edited is that it attempts to fill this gap. Nevertheless, the clearest point to emerge is how indirect our knowledge is of the effects of different kinds of social contact. Even the best chapters show this. Those, for example, by Roger Goodwin on

language and by Neil Warren on cross-cultural psychology are particularly impressive. Both are comprehensive and entertaining. But the chapter on language has virtually nothing on people talking to each other — rather odd in a book with society in its title — and the cross-cultural chapter is more concerned with connections between "primitive" and child-like mentalities than with the transmission of culture from one person and generation to another. The topics seem to be social, but the meat of social behaviour — social interactions — is left out.

A more glaring example of discrepancy between appearance (the chapter heading) and reality (what lies beneath) occurs in Wallace's chapter on educational competence. Nothing is said in it about teaching the child, who is left to get on with his own development without any help from anyone else. This abandonment reflects a Piagetian bias, which also dominates a chapter by Furth on Piagetian perspectives. Nothing social there, and very little in a chapter by Archer and Lloyd on sex differences. The closest one gets to social interactions is in an interesting though speculative chapter by Trevathan on very early physiological and social development, and by Isbell and McKee on social cognition.

None of the authors should be blamed too harshly for neglecting social interactions in a book on children in society. It is the fault of developmental psychology, which still has to get to grips with Winnicott's insight. □

P. E. Bryant is Watts Professor of Psychology at the University of Oxford.

CORRESPONDENCE

Continued from p. 8

This is not the ideal test of "survival of the fittest"; it does not compare fitness and survival within one population. Instead, it compares populations, of which the later is descended from survivors of the earlier (at both the individual and the species level). The populations are separated by some millions of generations; this interval is long enough for comparison of the populations with an objective standard to reveal an increase in fitness. Could this cumulative increase in fitness be produced by anything except "survival of the fittest"?

D.G. STEPHENSON

Department of Geology,
University of Keele, UK

1. Rudwick, M.J.S. *Br. J. Phil. Sci.* 15, 27-40 (1964).
2. Paul, C.R.C. in *Patterns of Evolution* (ed. Hallam, A.), 125-158 (Elsevier, Amsterdam, 1977).

University staffing

SIR — On behalf of my members I should like to make comment on the article, "Change wanted" in *Nature* of 11 June (p.442).

I do not wish to proffer an opinion on the second interim report from the Swinnerton-Dyer committee but I do want to protest most strongly about your suggestion that a cutback in non-academic staff would be quicker and should be considered first.

First, the financial savings accrued from such cuts would be a drop in the ocean in comparison with the salaries saved from the academic staff. We as technicians are aware of departments with an academic staff establishment which in no way reflects the actual number of students taught. It is the top-heavy nature of such departments that needs careful consideration.

Second, the last sentence in the article asks "But is not the university an institution whose chief purpose is academic?". In order to maintain that purpose the academics need the back-up services of trained technicians to provide an efficient lab class and assist with research projects. An academic with a heavy teaching load trying to do research at the same time would either have to cut back the amount of teaching or give up a substantial part of his research projects in order to replace the technical services now provided.

As regards cleaners and porters, they already work in rather grubby conditions and I am sure that academic standards would not be improved if the academics had to clean their own rooms or provide an adequate supply of toilet paper in the lavatories.

In conclusion, we are aware that because of government cuts, savings must be found somewhere. But please do not point the finger at one group of staff. Far rather let each college put its own house in order and safeguard the jobs and careers of all its employees by looking at other areas of saving first.

The universities could also make a positive and valuable stand against the government cuts in an effort to maintain the opportunity of higher education for as many people as possible.

A.L. PRICE THOMAS

Branch Chairman,
Association of Scientific, Technical
and Managerial Staffs,
Westfield College Branch, London NW3, UK

Psychiatry on trial

SIR — Your ill-disguised dismissal of R.D. Laing (*Nature* 4 June, p.367) does little to clarify the many interconnected issues raised by the Sutcliffe case. Permit me to draw the following lessons from his trial:

(1) Escape into the protection of some illness, however well-defined or spurious, is no longer possible. Each of us needs to accept responsibility for our actions.

(2) The utter "normality" displayed by Sutcliffe during his trial now puts the onus on psychiatry to defend its labelling of unacceptable behaviour as "illness": may I remind you that there are psychiatrists amongst those who collude in the incarceration of Soviet dissidents.

(3) That anyone, particularly psychiatrists, should be surprised when the "common and pervasive" sexual abuse of, and violence towards women takes such an extreme form, is but a sad reflection on our society. Where I beg to differ from you is in not ascribing this to "psychiatric illness" but rather to the inevitable consequence of a pervasive morality. The sooner we stop hiding behind the comfort of psychiatric illness the better we shall see our own responsibility as members of the society that has nurtured Sutcliffe.

K. PAULUS

Clifton, Bristol, UK

Search for truth

SIR — Although not directly involved in the investigation of the origin of species, astronomers are, nevertheless, involved at the "sharp end" of research into origins as they seek to explore the Universe and as such I would like to comment on the leading article "How true is the theory of evolution?" (*Nature* 12 March, p.75).

Darwin's theory of evolution, like the theory of special creation, is just that, a theory, which is incapable of being proved as fact by scientists, and also incapable of being falsified. Both theories therefore, if given the label of scientific theories, fulfil Popper's second criterion. Second, in order to assemble and evaluate evidence for particular theories, scientists, hopefully, try to be as objective as they possibly can; if not, then their credibility may well be called into question. However, most people would find it impossible to be totally objective and impartial in weighing up evidence. Each of us has prejudices which we are incapable of putting out of our minds as we seek to assess observed facts, so their interpretation can never be fully objective.

This problem is particularly acute when the origin of the species is being investigated. The whole question of the existence of God and as a consequence our accountability to him as God, past conflicts between church leaders and scientists, dissatisfaction with the implications of evolution on the one hand and with the role of the Bible and the church on the other have all made the investigation of the origin of the species a good deal more subjective than other areas of scientific investigation.

Creationists will do themselves a great disservice by choosing to bury their heads in the sand as scientific investigation proceeds in the future but equally so will evolutionists if they draw up behind a barrier of indignation at the thought of Darwin's theory never

achieving the status of fact.

Objectivity of investigation and interpretation is not only desirable but very necessary, for in the final analysis the truth will stand all investigations and still be the truth long after we are all laid to rest.

HOWARD READ

Department of Astronomy,
University of Manchester, UK

American Creation

SIR — The correspondence arising from the British Museum's cladistic activities has had one common aim — to avert the imminent threat of an upturn in creationism (Jukes, *Nature* 21 May p.186; editorial 28 May p.271). We are tempted to ask why evolutionism feels threatened by creationism, when the real controversy has not yet been stated openly. This does not lie in E. O. Wiley's question (*Nature* 30 April, p.730) "Does the phenomenon of evolution occur?", since the majority of creationists would not deny that evolution occurs, but in the question "Did the evolutionary mechanism provide the actual pathway from sterile Earth to living world?"

Any view of origins that does not invoke a supernatural Creator must conclude either that it did or that life arrived from space in some form, as proposed by Crick and Orgel, Wickramasinghe and Hoyle, and others.

Either view must be accepted by faith, either in the propositions themselves or in the ability of science to provide proof in the future. The atheist, whose metaphysical presuppositions do not allow him to countenance any form of divine activity, is more close-minded than many theists, who would happily accept either theory or the alternative, special creation.

To take a narrower view, any survey of Bible-believing Christians would reveal a wide spectrum of conclusions. These would range from those who believe that God has worked through essentially neo-Darwinian evolution, to those "creationists" who find current evidence for Darwinian evolution unconvincing and conclude that special creation is consistent not only with the scientific evidence but also with the whole of Scripture, the reliability of which can be verified experientially by the Christian.

It is often stated that many creationists take no account of the scientific arguments for evolution. Since the converse is also true, we will point to some of the issues we consider relevant. It is reasonable to point out that no plausible theoretical model exists which provides a mechanism for the spontaneous generation of nucleic acids as informational macromolecules specifying polypeptides which themselves mediate the replication and expression of that information. The experiments demonstrating the formation of a variety of organic molecules from presumptive prebiotic soups fall far short of providing a pathway for chemical evolution. Again, it is self-evident that the fossil record leaves much to be desired and few biologists recognise the dependence of the geological column on radiometric dating methods based on questionable assumptions about initial conditions. The whole history of evolutionary thought is littered with the debris of dubious assumptions and misinterpretations, especially in the area of fossil "hominids". To come up to date, protein and DNA sequence data, generally viewed as consistent with an

evolutionary explanation of diversity, are invariably interpreted using methods which presuppose, but do not demonstrate, evolutionary relationships, and which use criteria that are essentially functional and teleological. Finally, there is a collection of isolated fragmentary pieces of evidence which are usually dismissed as anecdotal because they are irreconcilable with the evolutionary model. From the above and other considerations it is possible to argue a strong case for special creation.

If, however, we are to be banished to the lunatic fringe, we find ourselves in the company of men who are regarded as the founders of modern science — Boyle, Newton *et al.* These men formulated the laws of physics and chemistry, not by invoking the supernatural but by assuming it, in the conviction that all things were made by an intelligent and all-powerful Creator who had imposed order and meaning on his creation. So it seemed to them, and is it not reasonable to ask whether they would have begun to ask the right questions if it had seemed otherwise? It has, for example, been suggested by Needham that the reason for the failure of the Chinese to develop the scientific method of the West was that they did not see order in nature as ordained by a personal, rational being.

Science can be conducted only within a context or "world view" which inescapably determines its aims and directions. We, as scientists, must be aware that as long as our society looks to the great god of science for direction and meaning, our own world view will be shaped by our own science. If we assert untruth, half-truth or hypothesis as fact, society and we ourselves will be misled and we will be responsible for the cultural, political and ethical consequences. Truth was never determined by majority opinion.

We are dismayed by the methods and attitudes of American Creationism. The legislature cannot be used to establish truth, either scientific or spiritual. Furthermore we believe that the Bible, though accurate, was not written as a scientific textbook but written primarily to lead men to a personal knowledge of God through His Son. In that knowledge we are able to say: "By faith we *understand* that the world was created by the word of God" (*Hebrews* 11.3).

CHRIS DARNBROUGH
JOHN GODDARD
WILLIAM S. STEVELY

University of Glasgow, UK

Room for faith

SIR — Contrary to what your editorial on creationism and evolution might suggest (*Nature* 28 May, p.271), few "card-carrying believers" within the scientific community would hold to the "God of gaps" theory you describe. Few see any major conflict between science and religion. Most see them as providing different languages to describe the same phenomenon — the view that God created the world and that evolution was the mechanism by which he did it is not contradictory. "God created the world" cannot be held up as a scientific theory. It is scientifically and theologically unprovable. It has to remain a matter of faith. However, some of the corollaries of creationism: the insufficiency of mutation and natural selection to account for intra-specific diversity, catastrophic geology, a shrinking rather than an expanding active gene pool; are worthy of

some consideration within a scientific context. Indeed, it is surprising how rapidly some tentative theories in support of evolution have become scientific facts. The absurdity of recapitulation and the half-truth of horse evolution are still incorporated into school textbooks as irrefutable fact. Evolutionists often present their "beliefs" as dogmatic "fact" in a similar vein to creationists.

What is required is not that creation should gain equal time within the school curriculum of Arkansas or the exhibitions of the British Museum (Natural History); but that school pupils and general public alike are presented with the solid facts as far as they are known with both their limitations and their certainties (but not with conjecture or extrapolation). And that they be encouraged to draw their own conclusions; to observe the data, to construct their hypothesis and to test it against the new data as they emerge. In other words, they should be encouraged to learn and apply the scientific method. This should be the basis of all biology courses and museum exhibitions.

However, the controversy will not be stayed by the application of scientific method; because what one believes about the origin of life and of man has a grave effect on how one treats fellow man and how society functions.

NEIL K. MCBRIDE

Microbiology Department,
Queen Elizabeth College, London W8, UK

Missing links

SIR — In your leading article "How true is the theory of evolution?" (*Nature* 12 March, p.75), you discussed the apparent inability of the Darwinian theory of evolution controlled by natural selection to explain the episodic nature of the fossil record.

It must be accepted that the fossil record of most evolutionary lines does indeed show changes taking place in discontinuous jumps rather than in a continuous manner. It is important to realize that even a continuous development resulting from the accumulation of many small changes may only rarely show itself as this in the fossils unearthed.

We are unlikely to find the fossil remains of more than one in a hundred million or so individuals. Most of the fossils which we see, therefore, are necessarily those of organisms which were common over large areas and which remained common and unchanged for long periods. For this to be possible the species concerned must have been well adapted to a particular niche in a stable environment. If the environment changes the niche must change, and may then be invaded successfully by another species, or even vanish altogether.

Once a species has filled a stable niche it can rarely gain by changing in anything but minor ways. In an environment which has been stable for a long time, all major neighbouring niches are likely to be filled by other successful organisms, so that individuals that differ from the species optimum are likely to be less successful than the norm. Natural selection will then act to *slow down* significant evolutionary change.

Rapid evolution can occur only where there is extremely intense intraspecific competition, as in the case of our own ancestors, or where a species is not well adapted to its habitat — for example in regions colder or wetter than the stable environment to which it was adapted, and/or with different fauna or flora. Population pressure in a successful species will constantly be supplying a trickle of individuals

to such non-optimal peripheral regions around the main habitat. In such a region a small mutation, useless or even mildly harmful, could be advantageous to survival outside the normal range. Again, this would be unlikely to help if the new area itself had a stable climax ecology where all important niches were already filled. It is in a small area with many empty niches, and perhaps cut off by some natural barrier introduced by climatic, volcanic or other phenomena, that a now non-optimally adapted species may survive and find a whole series of minor mutations advantageous, eventually leading to major changes in structure and behaviour. During the whole of this development numbers will be limited, allowing a significant degree of genetic drift, and the intermediate forms may not persist for long, so that the chances of our finding fossils of such intermediates will be slight.

Not until the species has changed so much that it can successfully invade a new stable area when the barriers break down, as the rabbit did in Australia, can it become common and persistent over an extended period — and give us a chance to find its remains.

I am not trying to disprove the occurrence at some times in the past of large discontinuous jumps in evolution, or to prove that all evolution has occurred as a result of natural selection acting on a large number of random mutations, mostly small. All I am saying is that whichever of these is true the fossil record must be expected to be discontinuous.

"Missing links" may be truly missing, or simply so small in number and period of existence that they have not been found.

J.H. FREMLIN

Department of Physics,
University of Birmingham, UK

Scientists for sale?

SIR — Increasingly, the members of the biomedical research community are being forced to give their activities a greater immediacy of application. The commercial pattern into which the work on genetic engineering and monoclonal antibodies is falling reflects this pressure. There could arise conflicts in the academic mind some of which merit public discussion.

In the past there have been no constraints, other than moral, on the free movement of individuals between research groups. However, now, many of the commercially useful activities, whether in industrial or non-industrial laboratories, involve team work. These groups, like football teams, require continuity for success and their members, it can be argued, should be subject to the same restraints on movement from laboratory to laboratory as are footballers between teams. Specifically, contracts of employment should be considered as not only binding on the employer but also on the employee. It could even be maintained that changes within the period of a contract could be arranged by mutual agreement but only on payment of an appropriate transfer fee. Breaking of the contract by either employer or employee should be subject to the process of the law in the usual way.

As things stand at the moment, poaching of staff and of ideas on which considerable development money has been spent can occur without let or hindrance.

A.J.S. DAVIES

Institute of Cancer Research,
Royal Cancer Hospital, London, UK